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STUDIES OF CELL MEDIATED IMMUNITY BY CELL MIGRATION INHIBITION

by

© VINCENT SIAO-HSIUNG LAO

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled STUDIES OF CELL MEDIATED IMMUNITY BY CELL MIGRATION INHIBITION submitted by VINCENT SIAO-HSIUNG LAO in partial fulfilment of the requirements for the degree of Master of Science in Immunology.

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Date: October 31, 1973

ABSTRACT

The phenomenon of cell migration inhibition, originally described as a manifestation of delayed hypersensitivity by Rich and Lewis (1932) was demonstrated by three methods indirect capillary migration, direct capillary migration and direct agarose migration. The inhibition of migration shown in all these techniques was due to migration inhibition factor (MIF) activity.

In studying the cell mediated response in postpartum multiparous women, it was shown that sensitization to histocompatibility antigens could be caused by immunization to paternal antigens through pregnancy and could persist after the disappearance of cytotoxic antibodies. Sensitization by multiple transfusion was also detected by the cell migration inhibition assays. One implication is that allograft sensitization could be detected by these techniques in the absence of detectable humoral sensitization.

Cell migration inhibition assays had the capacity to detect "blocking factors" which occurred in the sera of some sensitized individuals. Sera that inhibited the release of MIF frequently inhibited unidirectional mixed lymphocyte culture (UMLC) stimulation. Although there is significant correlation between MIF activity and cytotoxic antibodies as well as cell mediated lympholysis (CML) the specific mechanisms that cause MIF production is still unknown.

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I. INTRODUCTION

The immune system functions to maintain the integrity of the living organism. In vertebrates, lymphoid elements are concerned with the fine degree of discrimination between normal or self components and foreign components, or foreign antigens. The lymphocytes are divided into three main subpopulations: stem cells, or precursor cells; B, or bone marrow derived lymphocytes, which are involved in the production of humoral antibody; and T, or thymus derived lymphocytes which are involved in the generation of cell mediated immunity (Miller *et al*, 1971, Parrott, 1971). Both T and B cells carry immunoglobulin or immunoglobulin-like receptors on their surface (Greaves & Hogg, 1971) which may constitute the recognition units of the antigen reactive cells, though there is some controversy over the exact nature of receptors on T cells.

After stimulation with certain antigens, the antigen reactive cells of both T and B variety, collaborate or interact, to give rapid proliferation. These proliferating cells consist of three specifically differentiated effector cell types: antibody forming cells or AFC (Szenberg & Cunningham, 1968); the effectors of cell mediated immunity or programmed T cells (Davies *et al*, 1966, 1969, Parrott, 1971, Cooper, 1972, Cooper & Ada, 1972); and memory cells (Cunningham & Sercarz, 1971).

Experiments on passive transfer of transplantation immunity have demonstrated that graft rejection is mediated primarily by sensitized lymphocytes (Mitchison, 1954) rather than by circulating antibodies (Algin *et al*, 1957). Although circulating antibodies were claimed to participate in a few *in vivo* systems of allograft rejection (Hasek *et al*, 1968), others have shown that they often have an inhibitory effect on graft rejection (*vide infra*). Thus cell mediated immunity is recognized as constituting the principal mechanism for graft rejection although the precise way in which these effector cells lead to the final rejection of the grafted cell remains largely unknown. The purpose of this study is to develop assay systems applicable to detecting cell mediated immunity in man, and the effect of blocking antibodies after sensitization.

II. LITERATURE REVIEW

(A) Cell Mediated Immunity (CMI)

(1) Overview

Cell Mediated Immunity is defined as an immunological response manifested by interaction of antigen with specific lymphoid cells, rather than antibodies. Historically, the phenomenon of CMI received recognition first in the sphere of chronic infective disease and was early known as bacterial allergy or delayed cutaneous hypersensitivity to infection. The classical example is the intradermal reaction to antigen prepared from tubercle bacillus which develops in man or experimental animals infected previously with tubercle bacilli. There is no immediate effect but an inflammation becomes apparent which in the course of 12 to 24 hours results in an edematous area caused by mononuclear cell infiltration (Gell & Hinde, 1951, Goldberg *et al*, 1962).

Other common CMI responses are found in some forms of contact sensitivity, immunity to many viral, bacterial and fungal infections, homograft immunity, certain autoimmune diseases such as experimental allergic encephalomyelitis and immunity to neoplasm (W.H.O. Report, 1969). CMI is an immune state that can be transferred passively with lymphoid cells but not with serum (Landsteiner & Chase, 1942). Clinical and experimentally induced immune deficiency diseases demonstrate the existence of separate pathways for the induction, maintenance, and expression of cellular and humoral immunity (Valentine & Lawrence, 1971).

(2) Induction of Delayed Hypersensitivity

In general delayed-type hypersensitivity (DTH) occurs most commonly following infection of the host with certain live bacteria or viruses (Chase, 1965, Turk, 1967). The injection into animals of dead organisms, other than tubercle bacilli, cannot induce marked DTH. DTH to egg protein was induced if the antigen was injected into tuberculous foci. It was also found that dead tubercle bacilli produced much higher levels of sensitization if incorporated in oil. This led Landsteiner and Chase, as well as Freund, to produce CMI to specific antigens by incorporating them in an adjuvant of killed mycobacteria in oil, subsequently known as Freund's complete adjuvant (FCA).

DTH to protein antigen can be induced under certain circumstances without the use of mycobacteria. A small dose of antigen in the form of antigen-antibody complexes prepared in antibody excess (Uhr, 1966), or 1 μg or lower doses of antigen in oil administered intradermally (Uhr, 1966), stimulates development of DTH that persists for several days or weeks without development of detectable antibody. Lymphoid cells from these sensitized guinea pigs or humans will transfer DTH to the specific antigen to normal non-sensitive recipients (Uhr, 1966).

(3) Histopathology of DTH

Following the intradermal injection of antigen into animals with DTH to the antigen, there occurs an initial capillary dilatation and a perivascular entry of polymorphonuclear leukocytes (Gell & Hinde, 1951). With time there is a steady increase in the proportion of mononuclear leukocytes predominantly macrophages and lymphocytes, (Goldberg *et al*, 1962, Turk, 1967) to reach maximum extent at 24 hours as an area of palpable skin induration which begins to subside after 48 hours. Although variation in cell types occurs during different phases of these manifestations of cellular immunity, and from one species to another, the large number of mononuclear cells present during the height of these reactions distinguish them from the type of immunologic damage mediated by antibody.

Characteristic forms of tissue damage during this reaction are also distinguishable and can be classified (Waksman, 1962) as: (1) the invasive-destructive lesion, where infiltrating lymphoid cells are associated with focal areas of destruction of antigen containing parenchyma, often also observed in the rejection of tumor and skin, the lesions of auto-immunity and graft versus host reactions; (2) vasculonecrotic lesion of tuberculin reactions where fibrinoid or necrotic changes in blood vessel walls and adjacent parenchyma are seen with the usual perivascular mononuclear cell infiltrate and an involvement of polymorphonuclear leukocytes; and (3) the massive necrotic reactions often observed at sites of severe tuberculin reaction, which seems to be ischemic infarction.

(4) Transfer of Cell Mediated Immunity

DTH is distinct from antibody mediated reactions on the basis of the different method of induction, slow progress of the delayed lesion and the inability to be passively transferred with serum. By contrast this form of immunity can be transferred to guinea pigs by means of mononuclear cells (Landsteiner & Chase, 1942). Lymphoid cells from lymph nodes and spleens can passively transfer tuberculin hypersensitivity (Chase, 1945) and so can peripheral blood leukocytes (Stavitsky, 1948). In the mouse, peritoneal exudate cells (PEC) can induce much more intense CMI than cells from other sources (Asherson & Park, 1968). The duration and magnitude of the sensitivity after transfer is greater between members of inbred strains than between outbred strains (Bloom & Chase, 1967), since syngeneic cells are not rejected whereas allogeneic cells cause rejection.

The passive transfer of homograft immunity can also be detected after syngeneic transfer of lymphoid cells from specifically sensitized animals by the ability of the recipient to reject skin grafts from the original sensitizing donor in an accelerated fashion (Billingham *el al*, 1963, Wilson & Billingham, 1967). On the other hand lymphocytes from a sensitized animal cause a vigorous inflammatory response when injected intradermally into the original donor (Brent & Medawar, 1967). Lymphoid cells from skin sensitized animals are also able to neutralize the growth of chemically induced tumor cells, syngeneic with the skin sensitizing donor, when a mixture of the immune and tumor cells is injected into syngeneic sublethally irradiated mice (Klein *et al*, 1960).

Studies of the positive DTH skin reaction in animals passively sensitized with transferred cells have demonstrated that there is little if any preferential localization of the transferred donor cells at the specific test site (McCluskey *et al*, 1963). Using isotope labelled cells, the vast majority of the cells in the infiltrate is contributed by the recipient of the transfer (McCluskey *et al*, 1963, Turk & Oort, 1963). Similar observations have been made in the passively transferred homograft rejection in mice (Najarian & Feldman, 1962, McCluskey *et al*, 1963) as well as in rabbits (Prendergast, 1964). These findings indicate that in CMI reactions, a small number of specifically sensitive cells may recruit, direct, or alter the behaviour of a large number of nonsensitive cells. Further evidence for the participation of non-sensitive transfer-recipient's cells in the generation of the cellular infiltrate is provided by the finding that irradiation of the recipient, prior to the transfer of sensitive lymphoid cells, temporarily prevents the development of a positive skin test (Coe *et al*, 1966).

In man, hypersensitivity to tuberculin has been transferred to patients receiving a blood transfusion from a sensitive donor (Lawrence, 1969). Subsequent investigations revealed that DTH could, in fact, be transferred with extracts or lysates of lymphocytes (Lawrence, 1969). This active substance in the lymphocytes has been given the operational designation of transfer factor. Transfer factor from donors artifically sensitized to an artificial antigen transferred long lasting specific DTH to recipients who could not have encountered the antigen before (Maurer, 1961). Transfer factor prepared from leukocyte donor A, who had been immunized by skin grafts from B when injected into recipient C can induce accelerated rejection of grafts from B but not from indifferent donors (Lawrence, 1957, 1965, Lawrence *et al*, 1960).

It is logical to consider that the ability of intact cells to transfer DTH is due to their content of transfer factor but it must be stressed that transfer factor, though a protein of 2,000 M.W. is not an immunoglobulin, nor, until very recently, could it be shown to transfer cellular immunity, *in vitro* or in any other species than man (Lawrence, 1969). Lysates of sensitized cells have not been able to transfer the ability to produce specific humoral antibody even when the donors have been recently boosted (Zweiman & Phillips, 1970).

(5) Lymphokines

Transfer factor has been described by its activity as a specific substance for the induction of DTH, *in vivo*. It is also possible to define specific cellular responses to antigens by *in vitro* production of other soluble factors (Bloom & Bennett, 1966, David, 1966). These factors are referred to as lymphokines. They probably play an important role in the rejection of homografts (Medawar, 1959, Gowan & McGregor, 1965) in tumor immunology (Klein, 1966, Klein & Sjogren, 1966, Old & Boyse, 1964) and in resistance against certain infections (DeClercq & Merigan, 1970).

The first of these factors described is the migration-inhibition factor (MIF) (*vide infra*) (Rich & Lewis, 1943, George & Vaughan, 1962). Subsequently, using a variety of methods for the study of cellular immunity, many other supposedly different mediators have been detected from sensitized lymphocytes in various systems. In addition to MIF, these include: macrophage activating factor (Nathan *et al*, 1971); macrophage aggregating factor (Lolekhar *et al*, 1970); antigen-dependent MIF (Amos & Lachman, 1970); chemotactic factor for moncytes (Ward *et al*, 1970), for eosinophils (Cohen & Ward, 1971) and for neutrophils (Ward *et al*, 1970); lymphotoxins (Ruddle & Waksman, 1967, 1968, Kolb & Granger, 1968); colony inhibition factor (Lebowitz & Lawrence, 1969); proliferation inhibition factor (Green *et al*, 1970); skin reactive factor (Bennett & Bloom, 1968); blastogenic factor (Valentine & Lawrence, 1969); and interferon (Green *et al*, 1969).

Many of these substances have not been purified to any significant degree, but the molecular weight of several of these substances is much smaller than immunoglobulin (Table I). Some of the biological activities may be associated with separate molecules, others may be similar or identical molecules capable of reacting differently in several *in vitro* systems. Although the functions of these factors in the mediation of cellular immunity *in vivo* are not clear, they provide *in vitro* tools with which to study the molecular basis of cellular immunity.

(B) Transplantation Immunology

(1) Evidence of CMI

Homograft immunity is produced by immunological contact of the host to donor histocompatibility antigens. As with induction of DTH to bacteria, the injection of foreign leukocytes intradermally will induce homograft immunity much more effectively than the intravenous route (Lawrence, 1959). This active induction of homograft immunity can be measured by the acquired ability of the recipient to produce an accelerated rejection of either skin grafts (Billingham *et al*, 1963) or tumors (Wilson, 1967) from a donor. Additional evidence for the role of the lymphocyte in this reaction is provided by the ability of the lymph node cell from a sensitized guinea pig to initiate a vigorous inflammatory response when injected intradermally into the original skin graft donor whereas the serum has no effect (Brent & Medawar, 1967). Immunity to skin grafts can be transferred with cells from the regional lymph nodes draining the site of graft application (Mitchison, 1953, 1954, Billingham *et al*, 1954). In contrast, transfer of immunity with serum having high specific antibody levels has rarely led to allograft immunity, although successful demonstrations have been reported (Hasek, 1972).

In mice, antibodies to histocompatibility antigens are detectable on the third and fourth day after skin grafting (Jensen & Stetson, 1961) and cytotoxic anti-tumor antibodies can be demonstrated at the height of tumor allograft rejection (Gorer *et al*, 1959). Mouse neoplasms, especially in the form of single cells, are sensitive to cytotoxic antibodies (Gorer & Kaliss, 1959) and certain dissociated cell grafts such as leukocytes and hemopoietic grafts, are damaged by the action of antibody, but solid tissue grafts are not very vulnerable except when implanted into previously sensitized recipients with donor specific cytotoxic antibodies.

Histological examinations of primary graft rejection reveal the appearance of infiltrating mononuclear cells as early as the second or third day after transplantation, consisting of variable proportions of lymphocyte and macrophages as well as some plasma cells. Macrophages obtained from the peritoneal exudate cells of tumor grafted mice are able, when injected together with tumor cells into irradiated recipients, to suppress tumor growth (Bennett, 1965). Because of the evidence and similarity between delayed sensitivity and homograft reaction, CMI plays a very vital role in transplantation immunity.

(2) Serologically Defined Histocompatibility Antigens

Histocompatibility plays a key role in the fate of tissue grafts (Little & Johnson, 1922, Loeb, 1930, Snell, 1948) and for each animal species a single strong histocompatibility locus is demonstrable: the H-2 of mice (Snell, 1948, Shreffler, 1967) HL-A locus of man (van Rood & Erniss, 1968, Dausset *et al*, 1965, Cepellini *et al*, 1967), Ag-B (H-1) locus of rats (Palm, 1964), B locus of chickens (Jaffe *et al*, 1943, Crittenden *et al*, 1964) and DL locus in dogs (Thomas *et al*, 1950). These antigens are on all nucleated cells, which in some species, such as the mouse, include the nonnucleated red blood cells.

In man the ABO blood group factors on red cells are also histocompatibility antigens, though the other red cell antigen such as M, N, S, Kjel, Duffy and Cc, Dd, and Ee are not probably because they are not found on other human cells and because they are very weak immunogens. ABO incompatibility may be responsible for the acute rejection of kidney (Gleason & Murray, 1967) and skin grafts (Dausset *et al*, 1966). Long survival of the A1 tissues in O incompatible recipients have been reported for skin (Cepellini *et al*, 1966) and organ transplants (Gleason *et al*, 1967). There is some evidence that incompatibility for blood group P antigens (which are probably present on pareachyma cells as well) may affect skin (Cepellini *et al*, 1966) and kidney graft survival (Gleason *et al*, 1967). Other non HL-A antigen can be detected by platelet complement fixation (Curtoni *et al*, 1967). Some of these, for example, the K° and Zw antigens appear to be confined to the platelets (van der Weerdt, 1965) but the B1 antigen described by Shulman (Shulman *et al*, 1964) has been found on both granulocytes and lymphocytes with certain HL-A specificities. It is not clear whether granulocyte specific antigens NA_1 NA_2 are transplantation antigens (Lalezari & Bernard, 1966).

HL-A antigens are determined by two principal series of closely linked co-dominant alleles on a single pair of autosomal chromosomes: constituting the LA (or first) and the "Four" (or second) series. Each chromosome has an allele for both the first and the second HL-A subloci, so no cell has more than 4 HL-A antigens. The existence of this main HL-A locus governing several alleles was demonstrated by Bodmer (1966).

The concept of the existence of two loci in the HL-A region was first stated by Dausset (Dausset *et al*, 1965) and later confirmed by Kissmeyer-Nielsen *et al* (1968) and Singal *et al* (1968) and many other participants at the four HL-A histocompatibility conferences up to that at Evian in 1972. Ten antigens [HL-A 1, 2, 3, 9, 10, 11, 28, W29, W31, W32] have been shown to be components of the first locus, and fifteen other antigens [HL-A 5, 7, 8, 12, 13, 14, 17, 27, W15, W18, W22, W5, and W10] are demonstrable at the second locus (Histocompatibility Testing 1972) (Appendix E) where W stands for Workshop defined antigens which have not yet been given HL-A status. Appendix D lists the present HL-A antigen table and shows the pattern of cross reactivity that have been shown between them.

It was estimated, in 1970, that 11% of the alleles in the first locus and 21% at the second locus were still unknown (Dausset *et al*, 1970). Other loci seem to exist (Walford *et al*, 1969) but investigation of further loci is complicated by the existence of crossing-over of HL-A specificities. The presence of a third and possibly a fourth series of HL-A antigen supposedly determined by an allele at an HL-A sublocus other than the first and second site has been postulated (Walford *et al*, 1969).

The initial correlation suggesting that the mixed lymphocyte culture (MLC) (*vide infra*) was activated by differences of the main histocompatibility complexes (MHC) assumed in large measure that activation was due to differences for the serologically defined (SD) antigen. Bach (1972) has demonstrated the MHC difference which lead to MLC activation are at least in some cases separable genetically form the SD loci. There is evidence that a lymphocyte defined (LD) locus which cannot be detected serologically is present within the MHC (Bach *et al*, 1972) (*vide infra*).

(3) Immunological Enhancement

The term "immunological enhancement" was introduced by Kaliss to denote a phenomenon in which humoral antibody facilitate the growth of the antigenically foreign tumor cells which would otherwise be rejected (Kaliss, 1958). Such antibodies can be passively administered or their formation induced by active immunization procedures.

Immunologic enhancement is presently defined as specific abrogation or prevention of immune rejection by antibody or antigen-antibody complexes and is different from immunologic tolerance which is a state of partial or complete incapacity to respond to the immunologic stimulus of antigen (Dresser & Mitchison, 1968). Enhancement can be generally classified into 3 types: afferent, central, and efferent (Kaliss, 1958, Moller & Moller, 1966). In afferent enhancement humoral antibodies neutralize the immunizing ability of foreign antigens (van Rood *et al*, 1968). A classicial example of such enhancement comes from experiments performed by Snell *et al* in mice in which it was shown that lymph nodes draining the site of a grafted allogeneic tumors were less reactive to the H-2 antigens of the tumor if the mice were injected with hyperimmune antisera prepared against these antigens before the tumors were transplanted (Snell and Winn, 1960). Efferent enhancement is the phenomenon by which humoral antibodies bound to the antigen of target cells decrease the susceptibility of such cells to stimulate immunologically programmed effector cells which would otherwise destroy them. This phenomenon may explain why passively injected antibodies can faciliate growth of allogeneic tumors in animals which have been immunized against the tumor alloantigens (Cepellini *et al*, 1967, Amos & Kaliss, 1968, Moller & Moller, 1966). An analogous effect can be demonstrated *in vitro* in which cultiviated mouse tumor cells were found to escape the lethal effects of lymphocytes immune to the H-2 antigens of the tumor if they were first incubated with a hyperimmune serum prepared against these antigens (Moller, 1965). Thus, efferent enhancement and the Hellstrom's early concepts of "blocking" antibodies may be closely allied if not identical.

In the central form of enhancement, it is conjectured that antigen-antibody complexes act directly on the immunologically competent cells and specifically decrease their immunological reactivity (Kaliss, 1958, Moller & Moller, 1966, Takasugi & Hildeman, 1969, Safford & Tokuda, 1970). It is necessary to suppose that antigen is present to direct antibody to the specific clone of ARCs. Diener and Feldman have postulated that antigen antibody complexes may then build up a lattice structure on the membrane of specific antigen reactive cells so that they are inactivated immunologically (1972). Doses as low as 0.0005 ml of whole immune serum *in vivo* will protect an antibody resistant and highly antigenic immune tumor against the lytic effects of immune lymph node cells (Gorer, 1961, Batchelor *et al*, 1962, Hutchin *et al*, 1967). *In vivo* experiments by Amos (1970) showed clearly that ascites tumor cells and antibody could cause immunologic suppression of the host animal. He cautiously called the substance responsible for this ISS, or immunologically specific substance, which has characteristics of antigen-antibody complexes.

The role of antigen together with antibody is in accord with the observation by Stuart *et al* (1968) that minimal prolongation of rat kidney grafts occurs with administration of antibody alone, but that marked prolongation (or enhancement) was observed when antigen was also administered. This observation coincides with others that indicate some forms of allograft tolerance are caused by the formation of enhancing antibodies. In rats, immunological nonreactivity to transplanted "semiallogenic" rat kidney can be induced by inoculation of antiserum to the foreign alloantigen of the grafts although the rats continue to form antibodies against the foreign antigens of their grafts for a long time (Stuart *et al*, 1968, French and Batchelor, 1969, Rowley *et al*, 1969). Furthermore, supralethally irradiated canine chimeras possess both a cellular immunity and enhancing serum factor after allogeneic bone marrow transplantation,leading to the suggestion that a chimeric individual serum can protect against such cellular immune reactions that would otherwise lead to the destruction of its "own" cells (Batchelor & Howard, 1965).

In mice the most significant demonstration is that enhancing antibodies can contribute to the protection of the conceptus from immunological destruction by the mother's immune lymphocytes (immunocompetent cells) (Hellström *et al*, 1969). Maternal mice according to *in vivo* tests are specifically less reactive to allogeneic skin or tumor grafts carried by their offsprings (Hellström & Hellström, 1970).

(4) Pregnancy as a Model for Allograft Transplantation Study

Many hypotheses have been introduced to explain why the conceptus of a normal allogeneic mating is not rejected by the mother through immunological reactions against paternally derived alloantigens (Billingham, 1964, Currie, 1968). The most common explanation has been that the trophoblastic layer acts as a barrier which can "wall off" the embryo from the immunological system of the mother so that the mother neither becomes sensitized to antigens of the embryo, or, if sensitized, her immune response is unable to destroy the embryo (Broadbury *et al*, 1969, Currie *et al*, 1968, Currie & Bagshawe, 1968). There are many reports, however, which raise doubts as to whether the trophoblastic layer can be fully protective. First, morphologic data suggests that the rat trophoblast layer is not wholly continuous but has pores through which lymphocyte may penetrate (Tai & Halasz, 1968).

Second, there is evidence that newborn mice possess lymphocytes of maternal origin and that blood cells derived from a new born child occur in its mother's circulation after delivery (Tuffrey *et al*, 1969, Walknowska *et al*, 1969). Third, Soren applied the Simonsen graft-versus host assay to show that lymphocytes from mice of one strain which have undergone repeated pregnancies with a male of another strain are immune to the paternally derived antigen in the embryos (Soren, 1967). Fourth, in contrast to the last point several investigators have demonstrated that female mice which have been repeatedly impregnated by a male of another strain are specifically less reactive against alloantigens of the paternal strains than controls, and that such mice often possess haemagglutinating antibody to these antigens (Kaliss & Dagg, 1964, Breyere & Barnett, 1960b, 1961, 1963, Payne, 1962).

It has been suggested, therefore, that enhancement may play a role in pregnancy (Kaliss & Dagg, 1964). The independent demonstration by Payne and Rolfs (1958) and by van Rood *et al* (1959) that, in man, leukocyte antibodies are present in maternal serum following multi-pregnancies offers additional evidence of the occurrence and incidence of fetal-maternal transmission of leukocytes during gestation, since the antigens concerned are not present on erythrocytes nor in all probability are they present on trophoblast cells in an effective form (Seigler & Netzgar, 1970). Since multiparous women can only form antibodies against the leukocyte antigens transmitted to their fetus from their husband and absent in themselves, their antibodies are necessarily of limited specificity.

The evidence reviewed so far indicates that (1) cells from fetus do normally gain access to the maternal circulation and probably to the regional lymph nodes draining the uterus since they enlarge during heterospecific pregnancy (Beer *et al*, 1971); and (2) the mother is immunologically aware of and is, indeed, stimulated by the cellular antigen of her fetus.

(C) *In Vitro* Correlates of CMI

(1) Mixed Lymphocyte Culture

A variety of experimental studies have shown that at the peak of the immune response, the specifically active lymphoid effector cells comprise a surprisingly large number (Jerne *et al*, 1963, Wilson, 1965, Simmons & Fowler, 1966). Thus murine splenic cells producing hemolytic or hemagglutination antibody following immunization with sheep erythrocytes amount to 0.1-0.2% of the population (Jerne *et al*, 1963, Zaalberg *et al*, 1966), and the production of lymphocytes in the lymph nodes draining the site of a skin homograft which acquires specific cytocidal activities directed against the homologous target cells has been estimated to be 1-2% (Wilson, 1965). A similar proportion of adult fowl peripheral blood lymphocytes, when transferred into an allogeneic environment, are capable of producing pathological lesions - pocks on the chorioallantoic membrane; or splenomegaly - which are due immunologically to the graft-versus-host (GVH) reaction

(Simmons & Fowler, 1966, Simmons, 1967, Szenberg & Warner, 1962, Szenberg *et al*, 1962). The rate of the appearance of reactive cells in these various immunological systems has been shown to be essentially exponential.

A similar lymphocyte proliferation occurs *in vitro* under three conditions: (1) when cells from a sensitized animal or individual are exposed in culture to the sensitizing antigen excluding T antigens*; (2) when unsensitized cells are exposed to allogeneic T antigens* on an intact, or irradiated, or mitomycin treated cell (the two-way or one way mixed lymphocyte culture, or MLC); and (3) when unsensitized cells are exposed to certain substances, called nonspecific mitogens, such as phytohemagglutinin (PHA), conconavalin A (Con A) and poke weed mitogen (PWM). (* T antigens = transplantation antigens.)

Lymphocyte transformation *in vitro* can be defined simply as morphological enlargement of small lymphocytes to large lymphocytes (Robbins, 1964). The transformed cells morphologically resemble the pyraninophilic lymph node cells which appear after *in vivo* antigen stimulation (Parrot & de Sousa, 1966, Turk & Stone, 1963). This transformation may be due to a depression of transcription of inactive genes of the small lymphocyte which is preceded by an increased rate of histone acetylation (Pogo, 1966). Subsequently, the cells manifest a marked increase in their protein (McIntyre & Ebangh, 1963), RNA (Cooper & Rubin, 1965, Tanaka *et al*, 1963), DNA (MacKinney, 1963) synthesis culminating in mitosis (Nowell, 1960) of the transforming lymphocytes.

Regarding the kinetics of transformation, two major groups can be distinguished: (1) Lymphocyte transformation reaching maximal DNA synthesis after about 72-96 hours incubation. Stimulation of this type is achieved by phytohaemagglutinin (PHA) (Nowell, 1960), streptolysin (Hirschhorn *et al*, 1964) and leukocyte antisera (Grasbeck *et al*, 1963); (2) Lymphocyte transformation reaching maximum DNA synthesis about 144 hours after initiation of the culture. Antigen stimulation of sensitized cells (Pearmin *et al*, 1963, Hirschhorn *et al*, 1963, Ling and Hushand, 1964) and mixed lymphocyte culture (MLC) of unsensitized cells (Bain *et al*, 1964, Bach & Hirschhorn, 1964) belong to this category.

The number of original cells which respond in these lymphocyte transformations varies: 60%-80% of the cells are involved in the first type of transformation, whereas between .1% and 4% or original cells are involved in the second.

In 1963 Bain and her colleagues (Bain *et al*, 1963, 1964) described a proliferation interaction which resulted when leukocytes from immunologically disparate donors were mixed and placed into culture. Subsequent studies, using inbred strains of rats as cell donors, led to the conclusion that the reaction in the mixed lymphocyte cultures (MLC) represented an immunologically specific response by competent lymphocytes in cultures to homologous, but allogeneically different, cellular transplantation antigens (Wilson, 1967, Wilson *et al*, 1967c, Silver *et al*, 1967). It has been found that neontal thymectomy in mice substantially eliminates both PHA reactivity and responsiveness to alloantigens assayed *in vitro* in a host spleen cell population (Takiguchi *et al*, 1971) thus establishing that the cells which respond in MLC require the presence of T cells.

The MLC is similar to the primary phases of certain *in vivo* allogeneic lymphoid proliferations such as (a) the normal lymphocyte transfer reaction, which manifests itself as a delayed type of skin lesion, and involves the transformation of donor cells (Brent & Medawar, 1967), and (b) the cytology of the early phases of graft-versus-host reactions (Gowans & McGregor, 1965). The MLC is considered to reflect some of the afferent processes in transplantation immunity and supporting evidence for this view is that leukocytes from a 6-day MLC were able to confer accelerated graft rejection in rats (Gordon *et al*, 1967).

Stimulation in MLC tests (Bach & Voynow, 1966, Bach *et al*, 1970) is dependent on differences at the major histocompatibility complex. In the mouse, the major histocompatibility complex has recently been defined as including two serologically defined loci, H-2K and H-2D, the immune response (Ir) loci and the Ss-Slp locus; of these the locus for MLC stimulation seems to be within the Ir locus. In man, the two serologically defined subloci (LA and "Four" in the HL-A system) have been identified but the alleles of the LD locus responsible for MLC have not yet been defined. This locus segregates, within families, with the HL-A SD antigens as shown by the fact that HL-A identical siblings usually fail to stimulate each other in MLC. However, amongst unrelated individuals with identical HL-A SD antigens, the MLC is positive in 80% suggesting that MLC LD antigens are the cause. The fact that 20% of SD identical unrelated individuals fail to stimulate in MLC is attributed to "linkage disequilibrium" between certain LD and SD antigens. The fact that within families, sibling pairs differing by two HL-A hoplotypes stimulate approximately twice as much as those differing by only one allele (Albertine & Bach, 1968) is not incompatible with this hypothesis. This observation has been confirmed in both man (Sorensen & Kissmeyer-Nielsen, 1969, Schellekens & Eijsvoogel, 1968) and for AgB haplotypes rat (Wilson & Nowell, 1970). Each family haplotype is presumably linked to its LD allele, and two LD differences stimulate more than one, in MLC.

The importance of Ir genes in MLC has been established by studies with inbred strains of mice (Dutton, 1966) where responses in the MLC have been observed in cell mixtures that are identical at the H-2 locus but incompatible at the minor histocompatibility loci (Rychlikova & Ivanyi,(1969) and the Ir locus. In 1968 an exceptional case in which human siblings who were serotypically identical did respond to each other in the MLC has been reported (Amos & Bach, 1968). This is explained by crossing over between HL-A and MLC regions. In unrelated HL-A identical individuals Kissmeyer-Nielsen (Kissmeyer-Nielsen *et al*, 1970), Sorensen and Nielsen (Sorensen & Nielsen, 1970) and Schellekens (Schellekens *et al*, 1970) and their colleagues have studied eight, 20, and six unidirectional MLC, respectively and observed stimulation in all instances. The discrepant reactions are attributable to (1) the variant of certain well defined HL-A antigens e.g. HL-A 2, (2) unknown antigens possibly of a third sublocus or HL-A segregant series, or (3) LD different alleles on chromosomes that are SD identical.

The one way MLC (UMLC) (Amos *et al*, 1969), in which one population is treated with mitomycin or irradiation to prevent division, is now being used widely as both an index of cellular immunity or unresponsiveness. Accelerated but not increased MLC responses after allograft transplantation can be used to detect cellular presensitization (Miller *et al*, 1971). Inhibition of UMLC in the presence of the patient's serum have been demonstrated following a successful renal transplant (Hattler *et al*, 1971). IgG separated on DEAE columns from the transplant recipients is able to inhibit UMLC where donor lymphocytes are tested.

(2) Cell Mediated Cytotoxicity

Lymphocyte mediated cytotoxicity against various target cells in culture has been found to be particularly valuable for the understanding of cell mediated immune reactions in graft rejection. Lymphocyte mediated cytotoxicity against various target cells can be induced in culture by (a) the addition of lymphocytes previously sensitized against the corresponding target cells and (b) nonspecific mitogen, such as phytohemagglutinin, added to nonsensitized lymphocytes and target cells. Only in the first instance is the lymphocyte action immunologically sepcific. Cytotoxic reactions induced by nonspecific mitogen, (b) above appears to be nonspecific because the lymphocytes can be induced to kill autologous target cells (Lundgren & Moller, 1969). In certain experimental situation, when a pronounced degree of sepcificity was observed, it was claimed to have different specificity from that indicated by humoral antibodies (*vide infra*).

In the Colony Inhibition (CI) assay (Hellstrom, I. *et al*, 1965, Hellstrom, I., 1967) the suspension of target cells are seeded on petri dishes and later exposed to the lymphocytes to be tested for anti-target cell immunity. The ability of such lymphocyte to depress the colony-forming ability of the plated cell is measured. In human neoplastic diseases sensitized lymphocytes will not only kill fibroblasts from cancer patients but will be inhibited from killing in the presence of the autologous sera (Hellström & Hellström, 1969). This blocking effect may be analogous to the enhancement after allograft sensitization.

Direct cell mediated lympholysis (CML) assay using ^{51}Cr release from target cells (sensitizing population) is also an *in vitro* test of CMI which reflects *in vivo* sensitization. Sensitized lymphocytes are mixed with sensitizing lymphocytes to detect direct cytotoxic killing. The short (4 hour) period for incubation of attacking with target cells does not provide enough time for sensitization *in vitro*, and the expressed cytotoxicity represents the *in vivo* state of sensitization. CML is a thymus dependent and non-complement requiring process in which close cell to cell contact is a prerequisite (Brunner & Cerrottini, 1972). The reaction is immunologically specific (Miggiano *et al*, 1972). The mechanism of target cell injury is far from clear, but reports that 3', 5' cyclic nucleotides play a role suggests the involvement of a secretory process (Strom *et al*, 1972).

The relation of the cytotoxic properties of immune lymphocytes *in vitro* to graft destruction *in vivo* is also unclear. Thoracic duct cells (Wilson, 1965) from grafted animals could kill target cells *in vitro*, however, the insignificant number of radioactive labelled sensitized cells suggests other mechanisms like lymphokine release and recruitment of unsensitized cells may be operative *in vivo*. The CML *in vitro* reflects only one aspect of graft rejection *in vivo*.

(3) Migration Inhibition Factor (MIF)

In 1932 Rich and Lewis found that tuberculin inhibited the migration of cells from explanted spleen fragments, and caused buffy coat leukocytes to aggregate in plasma clots taken from animals with delayed hypersensitivity to tuberculin. They also noted some cytotoxic changes in these cells (Rich & Lewis, 1932). Other studies did not confirm their demonstration (Cruickshank, 1951, May & Weiser, 1956) but most studies were confirmatory (Holden *et al*, 1953, Carpenter, 1963). George and Vaughan (1962) introduced a modified cell migration assay. Oil-induced guinea pig peritoneal exudate cells (PEC) packed by gentle centrifuation into capillary tubes were allowed to migrate out into a flat surface. PEC from guinea pig exhibiting delayed hypersensitivity were markedly inhibited from migrating out of capillary tubes by specific antigen (David *et al*, 1964a, 1964b). In sharp contrast, cells obtained from animals producing serum antibodies only were not inhibited by the specific antigens. Attempts failed to passively sensitize PEC from normal animals by incubating these cells in serum from sensitized animals indicating the cytophilic antibodies were not involved. The inhibition occurred in the presence of heat-inactivated serum, ruling out participation of complement factors (David *et al*, 1964a, 1964b, 1964c).

Antibody-mediated hypersensitivity reactions can be evoked on challenge of the sensitized animals with the immunizing hapten conjugated to many unrelated proteins. These humoral reactions do not depend for their detection on the specificity of the carrier proteins used for immunization. In delayed hypersensitivity, however, there exists a major contribution by the carrier protein to the specificity of delayed reactions as described by Benacerraf and Gel (1959). Using the hapten-protein conjugates, DNP-ovalbumin, DNP-ovalbumin or DNP-bovine gamma globulin, David *et al* (1963c) demonstrated the obligatory participation of the carrier protein in determining the specificity of the inhibition of migration of PEC from animals immunized with the appropriate hapten protein conjugate, although immune serum would react with the hapten irrespective of the carrier protein.

The α-DNP-oligopolypeptide containing seven or more L-lysine (lysyl) residues elicit DTH (Stulbarg & Schlossman, 1968) whereas α-DNP-oligopolypeptide, containing 3-6 lysine residues, can only induce anti αDNP polypeptide antibodies. PEC cells from guinea pig with delayed hypersensitivity to α-DNP oligolysine (an average chain of 18 lysine residues) were allowed to migrate the presence of $\alpha DNP(lys)_3$, $\alpha DNP(lys)_4$, $\alpha DNP(lys)_6$ and $\alpha DNP(lys)_9$ and only the last antigen $\alpha DNP(lys)_9$ was able to inhibit cell migration (Schlossman, 1968).

The ability of puromycin and actinomycin D to reduce the inhibition and allow normal migration indicates that inhibition of migration is not the result of cytophilic antibody attaching to PEC and thereby stopping cell migration. Also, Amos absorbed out sera of high titer of cytophilic antibody to PPD with normal guinea pig PEC. These cells migrated normally in the absence of PPD, or when PPD was present. Similar experimental results were obtained for β-lactoalbumin and a cytophilic antiserum to that antigen. In contrast, however, cytophilic antibody passively sensitized (lung) alveolar macrophage to be inhibited in the migration on exposure to antigen (Heise *et al*, 1968). Also, if 7s and 19s fractions of rabbit anti sheep erythrocyte are mixed with normal PEC and sheep red cells, the cell populations containing the 7s (IgG) will be inhibited from migration, suggesting that cytophilic antibody may bind poorly to macrophages but very well to leukocytes and antigen antibody complexes may interfere with migration.

By separation of the cell population in PEC it is possible to demonstrate that it is the sensitized lymphocytes which in the presence of specific antigen release migration-inhibition factors (MIF) whereas the macrophages merely act as indicator cells (Bloom & Bennett, 1966). Purified sensitized lymph node cells when mixed with 95% of normal PEC can inhibit cell migration in the presence of specific antigens (David, 1966). When sensitized peripheral blood lymphocytes are cultured in the presence of antigens, they will release MIF into the medium and the cell free supernatant will cause inhibition of migration of normal PEC (Bartfeld & Kelly, 1968).

The migration technique has been adapted to human leukocyte migration assay as a measure of cellular hypersensitivity (Söborg, 1967). A number of workers have demonstrated a correlation between inhibition of migration and the presence of a positive delayed type skin reaction (Falk *et al*, 1970a, Falk *et al*, 1970b). The method has been employed for the study of delayed hypersensitivity to soluble (Mookerjee *et al*, 1969, Rosenberg & David, 1969) and particulate antigen (Söborg & Bendixen, 1967) and after kidney and liver transplantation (Eddleston *et al*, 1970) as well as skin grafting (Falk *et al*, 1970). MIF is heat stable (56°C 30') but cryolabile (Bartfeld & Kelly, 1968). When injected into a normal animal inflammation occurs that is similar to a positive delayed type skin test. MIF is a glycoprotein and the molecular weight ranges from 23,000 to 55,000 depending on the species that produces it and may also depend on the specific antigen that stimulates its production (Bloom & Bennett, 1971). However, MIF production can be triggered by mitogens from lymphoid cell as well as non-lymphoid cell lines (Tubergen *et al*, 1970, Photini *et al*, 1971). The molecular structure of MIF from mitogen stimulation is more heterogeneous than MIF from specific antigen stimulation (Remold *et al*, 1972). No one has determined the composition of MIF, nor have the amino acids of the protein moiety been sequenced.

The role of MIF *in vivo* is unclear. Tissue culture supernatant containing this activity have been shown to be leukochemotactic for mononuclear cell *in vitro* (Ward *et al*, 1969) and probably *in vivo* to induce vascular permeability upon injection *in vivo* (Maillard *et al*, 1972), and to evoke a delayed-type dermal reacting in guinea pigs following intradermal injection (Bloom & Bennett, 1968). It is plausible to consider that MIF participate in CMI, *in vivo*, perhaps serving as mediators for the attraction of cells from the circulation into inflammatory foci when released by sensitized lymphocytes that have encountered their specific antigen and perhaps conferring aggressor activity on these recruited cells in cytotoxic reactions (Grant *et al*, 1972).

Table I summarises some of the properties of different lymphokines.

Table Ia

Migration Inhibitory Factors*

SPECIES	Guinea Pig	Guinea Pig	Human	Human	Human
INDUCER	PPD, Tumor Ags	OCB-BGG, Con A	PPD,SKSD, PHA,PKM, Vaccinia	PPD,SKSD	None
CELL SOURCE	LNC,PXL	LNC	PBL	PBL	Lymphoblast lines
CULTURE TIME	6-96 h	24 h	72 h	72 h	4-6 h
TARGET CELL	GP-PX cells	GP-PX cells	GP-PX cells	CP-PX cells	Lymphoblast lines GP-PX
PROD.INHIB.BY	Mitomycin Puromycin	Actinomycin Puromycin			
M.W.(SEPHADEX)	25,000 & 55,000	35-55,000	50,000	55,000	
HEAT INACT.AT	80°C	80°C	80°C		
ELECTROPHOR.R_f		Pre-albumin			
DESTROYED BY:					
Trypsin	No (1 mg, insol)	Yes (4 mg, insol)			
Chymotrypsin	No (1 mg, insol)	Yes (4 mg, insol)			
Neuraminidase		Yes (100 ug)			
pH STABILITY	2-10				
LABORATORY	Bloom, Bennett	Remold, David	Thor	Remold, David	Glade, Broder

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[*] Bloom and Glade (1971)

Table Ib

Human Blastogenic and Potentiating Factors*

FACTOR	LTA	BF	CMRF	MF	EGO
INDUCER	PPD, DipTxd	MLC, Ags	None	PPD	None
CELL SOURCE	PBL	PBL	PBL adherent cells	PBL	Lymphoblast lines
CELL DENSITY	2.5×10^5	$1-2\times10^5$		10^7	10^6
CULTURE TIME	36 h	3-5 d	24 h	3 h	
HEAT INACT. AT	56°C				80°C
LABORATORY	Valentine, Lawrence	Janis, Bach	Bach	Maini, Dumonde	Adler, Smith

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* Bloom and Glade (1971)

Table Ic

Cytotoxic and Growth Inhibitory Factors*

SPECIES-FACTOR	Human-LT	Mouse-LT	Human-ClIF	Human-PIF
INDUCER	PHA (or none)	PHA,MLC	PPD,PHA	PHA,PPA,DipTxd
CELL SOURCE	Adenoids (or lymphoblast lines)	Spleen	PBL	PBL
CULTURE TIME	2 h-5 d	12 h-72 h	24 h	24 h
TARGET CELL	L-cells	L-cells	HeLa	HeLa,RB, HEP2
PROD.INHIB.BY	Puromycin,DNP, cycloheximide			
M.W.(SEPHADEX)	80,000 53,000 (sucrose grad.)	85,000		
HEAT INACT.AT	80°C	100°C	56°C	80°C
AMM.SULFATE	0-40% ppt.	60-80% ppt.		
DESTROYED BY	Phenol extract	Phenol extract		Trypsin
RESISTS	Trypsin, RNase	Trypsin, RNase		RNase, DNase
LABORATORY	Granger, Kolb	Granger	Lebowitz, Lawrence	Cooperband, Green Kibrick

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* Bloom and Glade (1971)

Table Id

Chemotactic Factors*

FACTOR-SPECIES	CF-GP	CF-GP	CF-Hu
INDUCER	oCB-BGG	oCB-BGG	SKSD
CELL SOURCE	LNC	LNC	PBL
CULTURE TIME	24 h	24 h	72 h
TARGET CELL	Rab. GP macrophages	Rabbit PMN	Rabbit, GP macrophages
M.W. (SEPHADEX)	50,000	50,000	50,000
HEAT INACT. AT	80°C		
ELECTROPHOR. R_f	α-Globulins	γ-Globulins	
LABORATORY	Ward, David	Ward, David	Ward, Rocklin

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* Bloom and Glade (1971)

Table Ie

Interferons*

FACTOR-SPECIES	IF-Hu	IF-Hu	IF-Hu
INDUCER	PHA,PKM	PPD,DipTxd	None
CELL SOURCE	PBL	PBL	Lymphoblast lines
CULTURE TIME	-	4+ days	
M.W.(SEPHADEX)	18,000		20,000
DESTROYED BY: Trypsin	Yes	Yes	Yes
HEAT INACT.AT	65°C	Stable at 56°C	Stable at 56°C
LABORATORY	Cooper, Grideman	Green, Kibrick, Cooperband	Kasel, Glade, Chessin

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* Bloom and Glade (1971)

Table If

Skin Reactive Factors*

SPECIES	GP	GP	GP	Mouse (PAR)
INDUCER	PPD	PPD,Con A PHA	Con A	MLC
CELL SOURCE	LNC	PXL,LNC	LNC	LNC, Spl
SUPERNAT.CONC.	10-20 X	1 X	1 X	10 X
SKIN TEST IN	GP	GP	GP	Irrad. hamster
INFILTRATE	Mononuclear, 4h	Mono. & poly	Mononuclear	Polys
PEAK REACT.	6-12 h	3-6 h	24 h	24 h
M.W.(SEPHADEX)	35-85,000	35-85,000		
HEAT INACT. AT		56°C		
DESTROYED BY:				
Trypsin	No (1 mg, insol)	Yes (10 mg, insol)		
Pepsin		Yes (250 μg)		
ELECTROPHOR. R_f		Pre-album. & albumin		
LABORATORY	Bennett, Bloom	Pick, Krejci, Turk	Schwartz, Leon	Ramseier

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[*] Bloom and Glade (1971)

Table Ig

Other Factors*

FACTOR-SPECIES	MAF-GP	IDS-Hu
INDUCER	PPD	None
CELL SOURCE	LNC	Lymphoblast lines
TARGET CELL	PEC	PBL
PROD.INHIB.BY	AcD Puromycin	
HEAT INACT.AT	80°C	56°C
M.W.(SEPHADEX)		10,000 or less
DESTROYED BY:		
Trypsin	Yes	
LABORATORY	Gotoff, Lolekha, Dray	Smith, Adler

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* Bloom and Glade (1971)

ABBREVIATIONS

AcD	actinomycin D
Ag	antigen
Amm Sulfate	ammonium sulfate
BF	blastogenic factor
CF	chemotactic factor
ClIF	cloning inhibitory factor
CMRF	conditioned medium reconstituting factor
Con A	concanavalin A
Dip Txd	diphtheria toxoid
DNP	dinitrophenol or dinitrophenylated
EGO	enhancer of gene operation
IDS	inhibitor of DNA synthesis
IF	interferon
LNC	lymph node cells
LT	lymphotoxin
LTA	lymphocyte transforming activity
MAF	macrophage aggregation factor
MF	mitogenic factor
MIF	migration inhibitory factor
MLC	mixed leucocyte culture
Mono.	mononuclear cells
OCB-BGG	*o*-chlorobenzoyl-bovine gamma globulin
PAR	product of antigen recognition
PHA	phytohemagglutinin
PIF	proliferation inhibition factor
PKM	pokeweed mitogen
PMN-or poly	polymorphonuclear leukocytes
PPD	purified protein derivative of tuberculin
PPL	peripheral blood lymphocytes
PX	peritoneal exudate
SKSD	streptokinase-streptodornase
SRF	skin reactive factor

III. MATERIALS AND METHODS

(A) Materials

(1) Lymphocytes

Peripheral blood leukocytes were harvested by the Ficoll Isopaque technique from (a) multiparous women [designated as (W)], (b) their husbands (H), (c) dialysis patients, (d) HL-A identical individuals and (e) control cells from a panel of normal individuals (C). The Ficoll-Isopaque solution is composed of (a) 33.9% solution of Isopaque (75% Sodium Metrizoat) (Laboratory Products Nyegaard and Co. As. Oslo) (b) 9% solution Ficoll (Ficoll, Pharmacia Fine Chemicals, Uppsala, Sweden). The final Ficoll Isopaque solution contains 13 parts of 33.9% Isopaque to 31.2 parts of 9% Ficoll with a specific gravity of 1.076-1.078. Whole blood or buffy coat layer, resuspended in Hanks medium was layered on top of the Ficoll-Isopaque solution in 1 = 1 ratio in a centrifuge tube. After being spun at 500 G for 35 minutes at 18°C the lymphoid cells were obtained by aspiration of the visible turbid zone in the Ficoll-isopaque mixture.

(2) Leukocytes

Peripheral blood leukocytes were obtained from randomly chosen volunteers with known HL-A profile by the Plasmagel sedimentation method. Heparinized blood was mixed in 3 to 1 ratio with Plasmagel.

Plasmagel is a product of Laboratoire Roger Bellow (159 Avenue de Roule, Neuilly, France) and contains 3 gms of modified gelatine 0.7 gm of NaCl, 0.2 gm of $CaCl_2 \cdot 2H_2O$ in 100 ml of distilled water. The mixture was allowed to sediment in a cylinder at 37°C for 40 minutes after which the leukocyte rich top layer was removed. The cells were then obtained by centrifugation for 10 minutes at 210 G. After 2 washes with Hanks medium, the cells were usually adjusted to about $40 - 80 \times 10^6$ cells/ml. To each ml of cell suspension 9 mls of freshly prepared 0.83% to NH_4Cl (Fisher Scientific Co.) was then added to lyse the red cells. Finally, the cells were washed twice in Hanks medium, and were counted and differentiated.

(3) Peritoneal Exudate Cells

30 ml of sterile light mineral oil (Fisher Scientific Co. viscosity 1.35) were injected intraperitoneally into 500 gm random bred guinea pigs. Approximately 72 hours later, the animals to death from the heart, under ether anesthesia, to reduce erythrocyte contamination of the peritoneal exudates. Cold Hank's balanced salt solution (Microbiological Associates, Bethesda, Maryland), 100 to 150 ml were injected intraperitoneally; the abdomen was kneaded gently, and the contents drained by means of a cannulated trocar and plastic tubing into a separatory funnel. The aqueous phase was separated from the oil and centrifuged at 300 G for 10 minutes at 4°C. If erythrocytes were present, the cells were treated with 0.83% NH_4Cl. The PEC are composed of 70% or more mononuclear cells (lymphocytes and macrophages),

the remainder being polymorphonuclear cells. After the cells were washed three times they were made up to 40 × 10^6 cells/ml in medium TC 199 (Hanks based) with 50 units of pencillin and 50 μg of streptomycin (Microbiological Associates, Bethesda, Md.) and 15% normal guinea pig serum.

(4) Serum

(a) Normal AB serum

(i) Peripheral blood from AB donors was taken by a donor set into sterilized glass tubes with rubber stoppers.

(ii) The blood was allowed to clot at room temperature for 6 hours then the clots were manipulated with sterile sticks to separate them from the sides of the tube.

(iii) The tubes were centrifuged 900 G at 4°C for 10 minutes and the serum on the top was removed by Pasteur pipets.

(iv) Sera from several AB donors were pooled together, aliquoted, and stored at -15°C.

(b) Autologous serum

(i) Autologous serum came from the peripheral blood of the donor who possessed the supposedly sensitized effector cell population.

(ii) The sera were aliquoted, dated, stored at -15°C and were decomplemented before use.

(c) Guinea pig serum

(i) Guinea pig serum was obtained from randomly bred guinea pigs by cardiac puncture after anesthesia induction with ether.

(ii) The blood was allowed to clot as above and the serum harvested in the same manner [4b(ii)]

(d) Fetal calf serum

Fetal Calf Serum was obtained from Microbiological Associates, Bethesda, Maryland in 100 ml bottles. It was aliquoted and stored at -15°C.

(e) Horse serum

(i) Horse serum was obtained as clotted whole blood from the University of Alberta Vivarium.

(ii) Serum was obtained, stored as above [4b(ii)].

(f) Decomplementation

All sera were decomplemented in a 56°C water bath for 30 minutes before experimentation.

(B) Methods

(1) Indirect Capillary Migration Test

(a) Human peripheral blood from husband, multiparous women, and unrelated persons was either taken by donor set (Cutter Laboaratories, Inc. Berkeley, California) into containers with 80 units of heparin per ml (Sodium heparin Injection USP, from intestinal mucosa, Connaught Medical Research Institute, University of Toronto, Canada) or by heparinized "Vacutainer" tubes (Bectow; Dickinson and Co. Rutherford, New Jersey).

(b) The heparinized blood was centrifuged at 500 G for 8 minutes. The buffy coat was removed and resuspended in 1 : 1 ratio with Hank's balanced salt solution (BSS). The cell suspension was then layered in a one to one proportion over Ficoll Isopaque solution.

(c) After centrifugation at 500 G for 40 minutes at 18°C, the cells at the interface were removed, mixed with an equal volume of Hank's BSS and centrifuged at 500 G for 10 minutes at 4°C.

(d) After two more washing with Hank's BSS the cells were resuspended in medium 199 with Hank's BSS, sodium bicarbonate, 100 units of penicillin and 100 µg of streptomycin per ml (Microbiological Associates Inc. Bethesda, Maryland) plus 15% of normal AB serum (ABS) or fetal calf serum (FCS).

(e) The cells were counted and differentiated in a haemocytometer. The procedure consistently gave a mononuclear cell population of 90%.

(f) Mixed lymphocyte cultures (MLC) were prepared by mixing 1 × 10^6 cells from each partner or 2 × 10^6 from one person in a total volume of 2 ml in TC 199 Hank based medium with 15% normal ABS or FCS. Cells were cultured in Falcon (12 × 75 mm, Oxnard, California) plastic tubes in a humidified incubator at 37°C in 5% CO_2 in air.

(g) Cultures were terminated on days 3 and 5. The cell free supernatant fluids from 3 and 5 day a MLC were obtained by pooling all the supernatants from identical cultures, centrifuging at 900 G for 10 minutes at 4°C and passing the fluid through 0.45 μ Millipore filter.

(h) The different groups of cell free supernatant were poured into dialysis tubing (Fisher Scientific Co. Pore size 10,000) and sealed at both ends.

(i) Each tubing was then dialysed against polyethylene glycol (MW 20,000 Fisher Scientific Co.) unitl 5 times concentrated.

(j) Then they were retightened at both ends, dialysed for 48 hours against 200 volume of Hanks BSS (with 50 units of penicillin and 50 μg streptomycin/ml), and the content was again dialysed for a further 24 hours with 50 volumes of fresher medium TC 100. Both dialysis steps were performed at 4°C and with a constant motion of the media by magnetic stirrers.

(k) The final osmolality of these supernatant fluids (ranged between 270-350 mom/kg) was checked by osmometer (Advanced Instrument Inc., Newton Heights, Mass.) and on a given experiment, osmolalities of the control and experimental supernatant fluid were approximately equal. These fluids were then assayed for presence of MIF activity by being used as culture medium on freshly harvested peripheral blood leukocytes from another unrelated person.

(2) Leukocytes as Indicator Cells

The migratory cell population was harvested by a previously described technique by Falk *et al* (1970) as follows:

(a) Peripheral blood was taken from an individual unrelated to those used for mixed cultures. After defibrination by glass beads, the blood was mixed in 3 : 1 proportions with plasmagel and allowed to sediment for 40 minutes at 37°C.

(b) The leukocyte enriched layer was removed and centrifuged at 210 G for 10 minutes in a refrigerated centrifuge, and the cell pellet was suspended in 1 ml of Hanks BSS per 10 ml of original whole blood.

(c) To this were added 9 volumes of 0.83 ammonium chloride, and after 3-5 minutes, the mixture was centrifuged at 200 G for 10 minutes. This procedure lysed most of the red cells.

(d) The cells were washed 2 times in Hanks BSS and resuspended in medium TC 199 with 15% normal AB serum. The cells were adjusted to a concentration of $30 - 40 \times 10^6$ cells/ml and packed into 20 µl capillary tube (Drummond Scientific Co.) of about 1 mm internal diameter and 6.5 cm long.

(e) The capillary tubes were cut at the cell-fluid interface and placed in a clear plastic chamber with a diameter of 2 cm.

(g) A small amount of silicone grease (Corning Co.) was used to maintain the tubes in place, and the chambers were filled with the concentrated supernatant fluids obtained after termination of 3 and 5 day MLC. A clear cover glass was used to seal the top of the chamber.

(h) After 18 hours of incubation at 37°C the chambers were placed on a 3M overhead projector (Minnesota Mining and Manufacturing Co.) and the image of cell migration was projected on the wall. The outline of the area of migration was traced onto a sheet of paper and the area was determined by a planimeter (Gelman Instrument Co.).

(i) In all experiments, at least four migration tubes were used for a given set and the average area of migration determined. The migration index (M.I.) was determined by dividing the average area of migration in experimental supernatant fluids (concentrated and dialysed MLC supernatants) by the average area of migration in control supernatant fluids from the unmixed lymphocyte culture containing the same number of lymphocytes from one person.

$$\text{MI} = \frac{\text{average area of migration in mixed culture supernates}}{\text{average area of migration of unmixed culture supernates}}$$

and a MI value of .79 or less was considered as a positive reaction and a demonstration of DTH.

(3) Mixed Lymphocyte Culture (MLC)

(a) Lymphocytes were harvested as by the Ficoll Isopaque method and cultured at a concentration of 1 × 10^6 cell/ml from each partner to a final volume of 2 ml or 2 × 10^6 cell/2 ml from one individual as previously described.

(b) Four hours before the termination of the 3 or 5 day cultures, they were pulsed with 1 μC ^{3}H thymidine per ml (specific activity 3.0 C per mM). The cell button was washed twice with 2.0 ml Beckmen scintillation fluid solution at 500 G for 10 minutes {3 liters of Beckmen toluene + 7.77 gm of 2,5 Dipenyloxazole (Amersham Searle) + 0.258 gm of 1,4 bis [2-(5 Phenyloxazlyl)] (Packard)}.

(c) The cells were then resuspended in 98% ethanol, spun at 900 G for 10 minutes and the supernatant discarded.

(d) 0.2 ml of 25% NCS (nuclear Chicago Solution) tissue solublizer in toluene (Amersham Searle Corp.) was added to each cell pellet and left for 30 minute at room temperature.

(e) A total of 9 ml of toluene scintillation fluid was added into each culture tube and the fluid was transferred into a screw cap scintillation vial (Beckmen Co.).

(f) All the vials were labelled on the caps and counted in a Scintillation Counter (Packard Model 3375).

(g) Blastogenic Index (BI) was determined by average counts per minute of the mixed cell culture divided by half the sum of the average counts per minute of one unmixed cell culture and the other unmixed cell culture.

$$\text{B.I.} = \frac{AB}{\frac{1}{2}(AA + BB)} .$$

(4) Unidirectional Mixed Lymphocyte Cultures (UMLC)

(a) Before setting up the cultures as described previously the stimulating cell population which is half the total cells (1×10^6 cells of each unmixed lymphocyte culture) were subjected to 7000 R of ^{137}Cs irradiation (620 r/min).

(b) The cultures were terminated and harvested in the same way as MLC described previously.

(5) Micro UMLC

(a) Peripheral lymphocytes were harvested by the Ficoll Isopaque technique as previously described.

(b) The final cells were suspended in Hanks BSS with 15% normal AB serum.

(c) The stimulatory cell population in the one way micro mixed lymphocyte culture (or half the micro unmixed lymphocyte cultures) were treated with 25 μg/ml Mitomycin C (Sigma Scientific Co.) for 25 minutes at 37°C. All cell populations were then washed 2 times with HBSS and the final cell concentration was adjusted to 1×10^6 cells/ml in Hepes buffered medium (Microbiological Associates, Md.) with 15% normal AB serum.

(d) The micro unidirectional mixed lymphocyte cultures were set up by mixing 100 μl of 1×10^6 cell per ml responder cells and 100 1 of 1×10^6 cells per ml Mitomycin C treated stimulator cell population, together into a microtiter well (Falcon Co.). If autologous serum was required 3 μl of serum was also added into the well.

(e) The cells were harvested by a multiple sample precipitator (Otto Heller, Madison, Wis.) on glass fiber filter paper.

(f) Each filter paper was taken out into a vial and counted in the scintillation counter.

(6) Direct Capillary Migration Test

(a) Peripheral lymphocytes were harvested by the Ficoll Isopaque technique as previously described.

(b) The lymphocytes from each individual were in 1 : 1 ratio MLC at a concentration of 2×10^6 cells/ml in TC 199 (Hanks base) medium with 15% pooled and decomplemented AB serum.

(c) After six hours of incubation at 37°C with 5% CO_2, the cultures were centrifuged at 500 G for 10 minutes and the supernatants were removed and stored.

(d) Each cell pellet was resuspended in its own supernatant at 30×10^6 cells per ml and packed into the 20 μl capillary tubes.

(e) The tubes were sealed at one by "seal-ease" clay centrifuged at 55 G for 6 minutes and cut at the cell fluid interface.

(f) These were placed in the 2 cm diameter plastic chambers which were filled by the respective culture supernates. A clear cover glass was used to seal the top of the chamber.

(g) The chambers were placed in an incubator at 37°C. After 18 hours the area of migration was determined by projection and planimetry. MI was determined by:

$$\frac{\text{average areas of migration of mixed cells}}{\text{average areas of migration of unmixed cells}}$$

In some experiments the MI was also determined by

$$\frac{\text{average area of migration of mixed cells}}{\frac{1}{2}\,(\text{average area of migration of 1 unmixed cells} + \text{average of migration of the other unmixed cells})}.$$

(7) Direct Agarose Migration Test - Preparation of the Agarose Plate

The agarose technique was first described by J.E. Clausen (Clausen, 1971) and was modified in the following way.

(a) Fresh agarose medium was prepared for each experiment. A 2% solution of agarose (J.T. Baker Chemical Co. N.J.) was prepared by weighing 2 gms of agarose per 100 ml of distilled water and dissolving the mixture in a boiling water bath.

(b) After cooling to 47°C, the 2% agarose medium was mixed at this temperature with horse serum (University Vivarium, University of Alberta, Edmonton) distilled water and ten-fold concentration of tissue culture medium 199 (Gibco) to give a solution containing 1% agarose, 10% horse serum in single strength of TC 199.

(c) Penicillin and streptomycin was added in a concentration of 100 units of penicillin and 100 μg of streptomycin per ml.

(d) Sodium Bicarbonate solution (7.5% $NcHCO_3$ from Microbiological Associates Bethesda, Md.) was added to that pH in the agarose medium after incubating in 5% CO_2, 95% atmospheric air saturated with water vapor was between 7.2 and 7.4.

(e) 6 ml agarose serum TC 199 medium was poured into Falcon disposable plastic petri dishes (Falcon, Bioquest Co.). After the gel had formed 6-7 holes of 2.3 mm diameter was cut in each agarose plate by stainless steel tubing, and the gel inside the holes was removed by suction.

(8) Application of the Agarose Plate

(a) Peripheral lymphocytes were either cultured for 6 hours or 24 hours in a 2 × 10^6 cells/ml concentration of MLC or unmixed culture as previously described.

(b) The cultures were spun at 500 G for 10 minutes and the supernatants were removed except about 50 µl left per 10 × 10^6 cells. The cells were resuspended by vibration. 10 µl lymphocyte suspension containing 2 × 10^6 cells was placed into every hole in the agarose plate (Oxford Pipets).

(c) In order to prevent drying up of the cells, 1/2 ml of TC 199 medium was placed evenly on top of the agarose plate.

(d) These plates were incubated for 21 hours and the area of migration was determined by projection and planimetry as previously described.

(9) Characterisation of MIF by Column Chromatography

(a) Sephadex G 100 (Pharmacia Fine Chemicals, Uppsala, Sweden) was first soaked in distilled water for 120 hours and then 72 hours in 0.01 M Na phosphate, 0.15 M NaCl buffer, pH 7.4 at 4°C.

(b) The Sephadex G 100 Phosphate Buffer Saline (PBS) mixture was poured into a 2.5 × 95 con glass columns (Pharmacia, Uppsala) with a 500 cc reservoir attached to the top.

(c) Cell free supernates concentrated 5-10 times by polyethylene glycol MW 20,000 (Fisher Scientific Co.) were first filtered by 0.45 μ Millipore filter and applied to the column.

(d) A LKB fraction collector (LKB Products Co. Sweden) and a LKB unicord III (Model 8300) flow spectrophotometer measuring @ 280 mμ were used in the cold room using a LKB recorder (Model 6520-3) for monitoring.

(e) Into each culture supernatant was added 5 mg of Chymotrypsinogen A. The supernatants were pumped through the column against gravity at 15 ml per hour and fractions containing 2.5 ml each were collected.

(f) Effluent fractions which appeared before protein of high molecular weight such as γ globulin were pooled and referred to as G-1. Fractions containing γ globulin were pooled and referred to as G-2. Fractions containing albumin were pooled as G-3. Fractions containing the chymotrypsinogen A indicator were pooled as G-5 and fractions between G-5 and G-3 were pooled as G-4.

(g) The pooled fractions were lyophilized (Virtis Research Equipment, N.Y.) and each pooled fraction was reconstituted 50 fold the original supernatant, dialysed for 72 hours against distilled water and stored at 4°C.

(10) Migration of Guinea Pig Peritoneal Exudate Cells

(a) Guinea pig peritoneal exudate cells were harvested by flushing the cell suspension out with HBSS 3 days after intraperitoneal oil injection. These cells, consisting mainly of macrophages and lymphocytes, were packed into 20 µl capillary tubes. The tubes were centrifuged at 50 G for 6 minutes and cut at cell-fluid interphase as previously described.

(b) To determine MIF activity, 20% of the aliquot of each pooled fraction was made up with TC 199 and 20% guinea pig serum were used as migration medium.

(c) The area of migration was measured by projection and planimetry. At least 4 capillary tubes were used for each sample.

(11) Autologous Sera of the Multiparous Women

(a) 1.5% decomplemented autologous sera of the multiparous women were added into each additional culture of HW, CW, and WW to make up a total of 15% serum concentration in TC 199 medium.

(b) To determine specificity of the autologous serum, serum from another multiparous women of the same concentration was added and into 3 other identical sets of HW, CW, and WW cultures.

(c) After six hours of incubation, the cells were harvested either for the direct capillary assay or the direct agarose migration assay to detect MIF activity.

(12) Rabbit Anti-Human Gamma-Globulin

(a) In some experiments, 3 concentrations (1, 10 and 100 µl/ml) of rabbit antihuman gamma globulin (Miles Lab. Inc. Ill.) was added into 3 sets of cultures before incubation, instead of autologous serum.

(b) After 6 or 24 hours the cells were applied into the wells of the agarose plate for detection of the migration area.

(13) Cytotoxic Antibodies and HL-A Profile

Both the cytotoxic antibodies and the HL-A profiles of the subjects were detected by the Tissue Typing Laboratory of the University Hospital, University of Alberta. The techniques were described by Terasaki and McClelland (1963) and by Mittal *et al* (1968). [Appendix A].

(14) Direct Cell Mediated Lympholysis (CML)

Direct CML was the work of Dr. T. Kovithavongs and Dr. J.B. Dossetor of the MRC Transplantation Unit, University of Alberta according to the techniques described by Wunderlich *et al* (1972) and Garovoy *et al* (1973). [Appendix B].

(15) Colony Inhibition (CI)

Colony Inhibition was the work of Dr. E. Liburd and Dr. J.B. Dossetor of the MRC Transplantation Unit, University of Alberta according to the technique described by Hellström and Hellström (1965, 1966) and Takasugi and Klein (1970).

IV. RESULTS

(A) Detection of MIF in Supernatant fluids of 5 day MLC Between Unrelated and Non-presensitized Individuals

MLC between 2 unrelated and non presensitized individuals, (Table II and III) were set up in medium 199 with 20% of either (1) pooled AB serum or (2) FCS, and terminated on day 5. The supernatant fluids were removed, concentrated, dialysed and the MIF activity of these supernatants tests using the culture fluids as migration medium for fresh human peripheral blood leukocytes. Supernatant fluids from 5 day mixed lymphocytes cultures were strongly inhibitory when compared with the control unmixed cultures supernatants. Remarkably similar results were obtained when cultures were set up with either pooled AB serum (MI = 0.53 ± 0.30 s.e.) or FCS (MI = 0.45 ± 0.07 s.e.). It is concluded that 5 day MLC between normal unsensitized cells produce MIF whether cultured in AB serum or FCS.

(B) Determination of Blastogenic Index (BI) in 5 day MLC between Unrelated and Non-presensitized Individuals

Lymphocyte cultures were set up between unrelated and non-presensitized normal adults in medium 199 with either 20% of (1) pooled ABS or (2) FCS. On day 5, triplicate cultures from each group were terminated after 4 hours incubation with tritiated thymidine. The

Table II

Indirect Capillary Migration in Supernates of

5 day Cultures with 20% Pooled ABS and BI

Migration Index (Planimetry units**)		Blastogenic Index
	0.43 (48/109)	19
	0.42 (61/146)	17
	0.36 (68/189)	19
	0.62 (90/144)	9
	0.55 (63/117)	11
	0.62 (20/32)	14
	0.57 (70/122)	12
	0.61 (128/210)	ND*
	0.62 (74/117)	14
MEAN	0.53±0.03 s.e.	14.4

* ND = Not done.

**Figures within parentheses indicate absolute areas of migration in planimetry units (Gelman Instrument Company Planimetor, Ann. Arbor, Mich. U.S.A. 1000 units = 1 sq. in.) Projection of migration chambers was done by a 3M overhead projector (see text).

Table III

Indirect Capillary Migration in Supernates of

5 day Cultures with 20% FCS and BI

	MI	BI
	0.64 (31/48)	7
	0.19 (16/85)	16
	0.32 (27/83)	12
	0.61 (39/65)	6
	0.58 (14/22)	ND[*]
	0.21 (13/66)	7
	0.61 (64/105)	3
MEAN	0.45 0.07 s.e.	8.5

[*] ND = Not done

cultures were harvested, digested and counted individually in a scintillation counter for 5 minutes. Only if the counts of the three vials in each group gave a standard error of less than 5% was that experiment considered valid. The mean BI for 5 day MLC with 20% AB serum is 14.4 and with FCS is 8.5. BI determination was carried out concurrently with experiments for the detection of MIF (Table II and III). It is concluded that there is no close inverse correlation between MIF as measured by MI and the BI.

(C) Detection of MIF in Supernatant Fluids of 5 day UMLC Between Unrelated and Non-presensitized Individuals

Sensitization was also detectable in 5 day one way MLC. UMLC were set up after irradiating cells from one party with 7000 R by Cs irradiation (^{137}Cs irradiation at 650 R per minute, Picker Nuclear). Controls contained equal mixtures of irradiated and untreated cells from the same individuals. Cell free supernatant fluids were treated in the same fashion as described previously. The migration cell population was peripheral blood leukocytes taken from either (1) an unsensitized donor unrelated to the donors of the MLC or (2) the donor who provided the allogeneic stimulating, but irradiated, cells in the mixed culture or (3) the same donor who provided the responding cell population.

The MI from the concentrated 5 day MLC supernates were (1) 0.38 using a 3rd and unrelated individual as donor for indicator cells,

(2) 0.40 using the stimulator's leukocytes as indicator cells and (3) 0.40 using the responder's leukocytes as indicator cells (Table IV). It is concluded that the origin of the indicator cells is not important in the detection of MIF.

(D) Demonstration of Direct Migration-inhibition and Blastogenesis in Cells after 5 day MLC and UMLC

After 5 days of culturing, triplicate cultures from each group were terminated after 4 hours incubation with tritiated thymidine, and later counted in a scintillation counter for determining the BI. The other cultures were centrifuged and the supernates were removed, concentrated, dialysed and stored. The cells from the MLC and UMLC were washed twice with medium 199 and 20% ABS, adjusted to a concentration of 40×10^6 cells/ml, and packed into the capillary tubes which were allowed to migrate in medium 199 plus 20% ABS. Both mixed cell populations, after 5 day MLC or UMLC,demonstrated migration inhibition compared to unmixed cell populations, and the MI of 0.33 and 0.42 from the two populations of cells was not significantly different (Table V). Blastogenesis were apparent in both 5 day MLC and UMLC cultures (Table V). It is concluded that the direct migration of cultured cells (MLC and UMLC) is inhibitable because of MIF being released in the mixture of cells though antibody mediated inhibition of migration is not excluded. There is also no close inverse correlation between MI and BI.

Table IV

Indirect Capillary Migration to Demonstrate MI of 5 day UMLC Supernates by Different Cells from Different Donors (A, B, & C)

MI	INDICATOR CELLS	unrelated	responder	stimulator
		C in $\frac{(AB^*)}{(AA^*)}$	A in $\frac{(AB^*)}{(AA^*)}$	B in $\frac{(AB^*)}{(AA^*)}$
		0.57 (49/86)	0.37 (36/99)	0.38 (31/82)
		0.23 (17/77)	0.28 (17/63)	0.39 (30/78)
		0.46 (28/62)	0.43 (21/51)	0.26 (12/47)
		0.36 (14/39)	0.58 (26/46)	0.53 (29/55)
MEAN		0.38±0.06 s.e.	0.40±0.05 s.e.	0.40±0.06 s.e.

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* 7000 R irradiation dose to stimulator cells in unidirectional MLC

Table V

Comparison of MI and BI after 5 day MLC and UMLC
(MI Determined by Indirect Capillary Migartion)

	MLC		UMLC	
	MI	BI	MI	BI
	0.33 (33/102)	19	0.40 (23/58)	7
	0.34 (21/62)	3	0.53 (40/77)	5
	0.29 (26/93)	8	0.62 (71/115)	17
	0.64 (53/84)	15	0.38 (27/73)	13
	0.19 (13/72)	13	0.17 (13/78)	8
	0.37 (32/89)	4	ND[*]	ND
	0.39 (29/76)	9	ND	ND
MEAN	0.33±0.06 s.e.	10.1	0.42±0.08 s.e.	10.0

[*] ND = Not done

(E) A Search for MIF in Supernatant of 3 day MLC between Unrelated and Non-presensitized Individuals (Table VI and VII)

Supernatant fluids from 3 day mixed lymphocyte cultures, having either (1) pooled ABS or (2) FCS as nutrient, did not significantly inhibit leukocyte migration. The MI of 1.01 from ABS-MLC and 0.99 from FCS-MLC is not significantly from unity. It is concluded that supernates from 3 day MLC (in contrast from those 5 day cultures) do not contain MIF, whether cultured in AB or FC serum. Figure 1 and 2 summarise the MI of 3 and 5 day studies in the AB serum or FCS between unrelated normal individuals' cells in MLC.

(F) Determination of BI in 3 day MLC between Unrelated and Non-presensitized Individuals (Table VI and VII)

Lymphocyte cultures were terminated on day 3 after a 4 hour incubation with tritiated thymidine. BI from cultures in pooled ABS and FCS were significantly high. It is concluded from experiments E and F that significant blastogenesis may occur in MLC without there being detectable MIF in the supernatant, in 3 day cultures of unsensitized normal cells.

Table VI

Indirect Capillary Migration in Supernates of 3 day MLC with 20% ABS and BI

	MI (planimetry units)	BI
	0.79 (87/100)	6
	1.44 (36/25)	5
	0.70 (96/137)	12
	1.15 (31/27)	4
	0.90 (43/49)	10
	1.05 (158/151)	10
	0.91 (83/92)	8
	1.14 (85/72)	ND*
	1.05 (99/95)	10
MEAN	1.01±0.07 s.e.	8.1

*ND = Not done

Table VII

Indirect Capillary Migration in Supernates of 3 day MLC with 20% FCS and BI

	MI (planimetry units)	BI
	1.09 (88/81)	2
	0.79 (101/136)	14
	0.69 (94/136)	10
	1.00 (38/38)	ND*
	0.93 (71/76)	ND
MEAN	0.99±0.07 s.e.	8.6

* ND = Not done

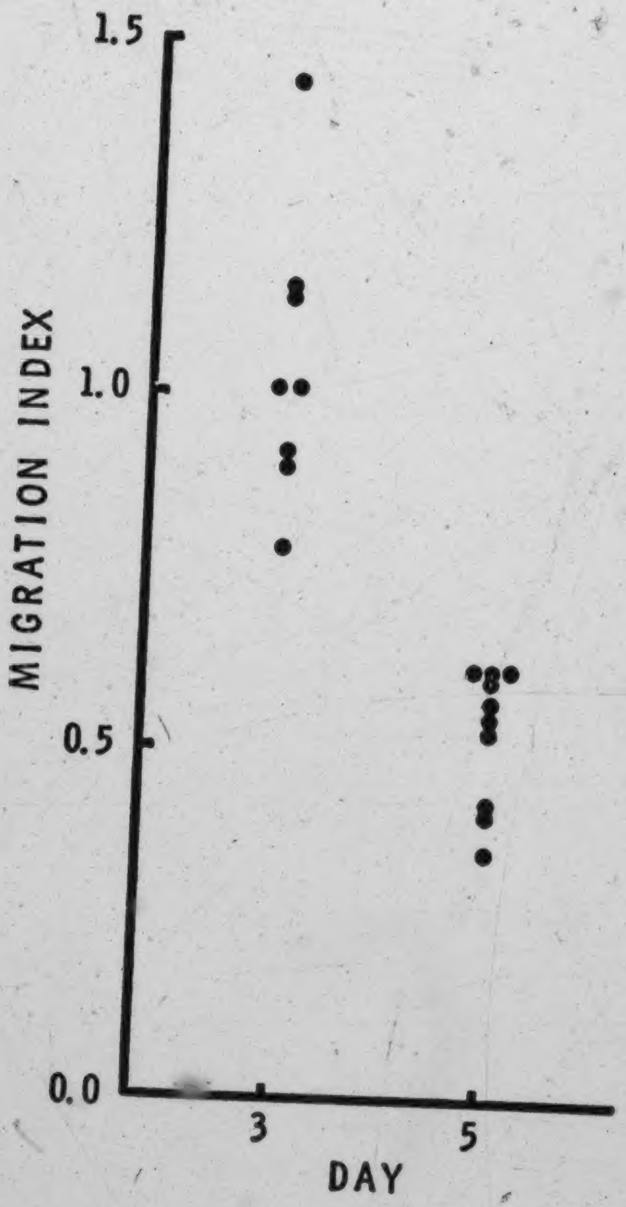

Figure 1 Indirect capillary migration in supernates of 3 and 5 day MLC of unrelated individuals in AB serum

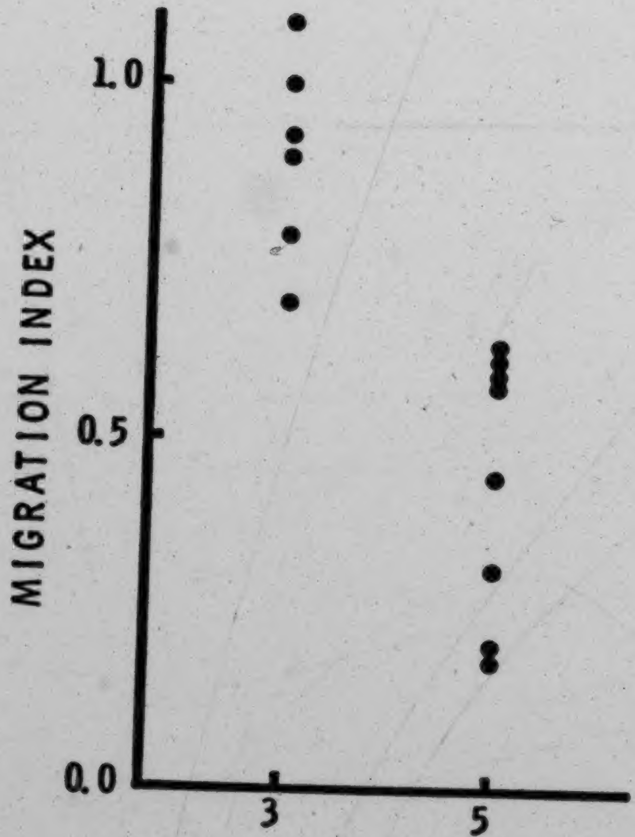

Figure 2 Indirect capillary migration in supernates of 3 and 5 day MLC of unrelated individuals in FCS.

(G) Detection of MIF in 3 day UMLC Supernates

Cell free supernates from the unidirectional lymphocyte cultures had a migration index of 0.91 ± 0.08 s.e. in six experiments, when a third individuals' peripheral blood leukocytes were used as the migratory cell population. With responder leukocytes migrating in concentrated supernates the MI was 1.12 ± 0.26 s.e. When the stimulator cells were the migrating cells and MI was 1.01 ± 0.05 s.e. (Table VIII). There was no migration-inhibition in any experiment, with one exception. It is concluded that 3 day UMLC supernates do not contain MIF when cells from an unrelated persons or from the donor that provide the UMLC cell are used as a migratory population.

(H) Direct Migration and Blastogenesis after 3 day MLC between Non-presensitized Individuals

After 3 days of MLC, the cells were washed twice with TC 199 and resuspended at a concentration of 40×10^6 cells/ml in TC 199 with 20% ABS. The cells were allowed to migrate in the same medium, and there were no inhibitions in any experiments (Table IX) despite significant increase of blastogenesis as demonstrated by BI.

Table VIII

MI, of 3 day UMLC Supernates, Using Three Types of Indicator Cells (C, A, and B) in the Indirect Capillary Migration Technique with Culture Cells from A & B

MI	unrelated	responder	stimulator
	C in $\frac{(AB^*)}{(AA^*)}$ supernates	A in $\frac{(AB^*)}{(AA^*)}$ supernates	B in $\frac{(AB^*)}{(AA^*)}$ supernates
	0.93 (63/68)	0.95 (76/81)	0.95 (68/72)
	0.94 (35/38)	0.73 (18/26)	1.11 (41/37)
	1.07 (93/87)	1.42 (164/116)	1.06 (98/93)
	0.98 (75/77)	1.13)80/71)	0.99 (76/77)
	1.16 (62/52)	0.99 (38/39)	0.93 (38/41)
	1.20 (62/52)	ND**	ND**
MEAN	0.91±0.08 s.e.	1.12±0.26 s.e.	1.01±0.05 s.e.

** ND = Not done

* 7000 R

Table IX

MI of Cells after 3 day MLC by Direct Migration and BI

MI	BI
0.91 (71/78)	3
1.09 (101/93)	17
0.89 (105/119)	14
0.93 (159/171)	ND*
MEAN 0.98±0.04 s.e.	11.3

* ND = Not done

(I) Detection of Migration of Cells from 3 and 5 day UMLC with the Addition of Fresh Leukocytes (Table X)

After cells from the 3 or 5 day UMLC were washed, they were resuspended in Hank's 199 with 20% ABS and counted. To each population of cells freshly prepared leukocytes from the stimulator donor were added in 1 : 1 ratio so that the final concentration of cells was 40×10^6 cells/ml. Cells were then packed into 20 μl capillary tubes and allowed to migrate in medium 199 containing 20% ABS. Most of the mixed cells from 3 day UMLC did not significantly inhibit migration compared with mixed cells from 5 day UMLC which showed significant inhibition in 4 out of 5 cases (Table X). It is concluded that a deficiency of stimulator cells in the direct mixed cell migration is not the reason for the failure of MIF detection after 3 day UMLC when normal unsensitized cells were used (nor did the extra stimulator cells distort the detection of MIF in 5 day UMLC direct mixed migration population).

(J) Detection of MIF in Supernates of 3 day MLC between supposedly Presensitized Individuals, Using the Indirect Technique

Multiparous women frequently have humoral antibodies in their serum direct against one or more of the antigen of their husbands. The purpose of this study was to see if CMI to the histocompatibility antigens of the husbands could be demonstrated in such women. Sera

Table X

MI of Cells from 3 and 5 day UMLC with the Addition of Fresh Leukocytes from the Stimulator

	UMLC	
	3 day	5 day
	0.95 (66/69)	0.40 (58/146)
	1.00 (73/73)	0.98 (125/128)
	0.73 (35/48)	0.33 (10/32)
	0.96 (82/86)	0.30 (29/97)
	0.85 (86/102)	0.33 (43/132)
	1.24 (146/118)	ND
MEAN	0.99±0.07 s.e.	0.41±0.12 s.e.

* ND = Not done

from multiparous women were obtained from the obstetrical service, and screened for the presence of antibodies against a panel of 5 cell donors, using Terasaki's microcytotoxicity test. MLC were set up between the cells of the wife with that of the husband (HW) and between cells from the wife with that of a random unrelated individual (CW). Controls contained cells from the wife only (WW) under identical cultures conditions. In six experiments, the unrelated third person (C) did not share any HL-A antigens with the husband (H) while in two experiments the unrelated third person (C) shared only one antigen with the hushand (H). A fourth person's peripheral blood leukocytes were used as the migration population in the concentrated supernates from (HW), (CW), and (WW) lymphocytes cultures.

The results are summarized in Figure 3. Significant MIF activity was detectable in the supernates of cultures between (HW) cells by the third day. Significant MIF activity was not detectable on the third day in the supernates of MLC between (WC). The mean MI of the supernatant fluids from 3 day MLC between husband and wife was 0.49 ± 0.04 s.e., whereas that for the supernatants from 3 day MLC of wife's cell and the cells from a third unrelated individual (CW) was 0.94 ± 0.06 s.e. The difference between these means is highly significant ($p < 0.01$). The BI, however, did not appear to be different between each group, (HW) and (WC), within each experiment (Table XI, Figure 3). It is concluded that although BI are increased at day 3 in HW and CW MLC, the supernatant by the indirect technique shows MIF only in the HW cultures.

Table XI

Indirect Migration of 3 day MLC
of Presensitized and Unrelated Individuals

Expt (RELATION)	HL-A	BI				OSMOLARITY		MI			
		3*		5*		3*	5*	3*		5*	
		HW	CW	HW	CW			HW	CW	HW	CW
CLAR (H)	(1)(5,8)	9		16		310	289	0.69		0.47	
(W)	(10,11)(W17,W18)					312	292				
HAYS (C)	(11,7)(12,W5)		12		30	293	296		0.79		0.43
SMIT (H)	ND	10		10		288	289	0.64		0.47	
(W)	ND					274	277				
ZIEG (C)	(2)(7 27)		10		11	298	292		0.90		0.55
GRAH (H)	(2,11)(5,W5)	16		21		293	316	0.40		0.50	
(W)	(1,3)(7±W22)					301	320				
OSLU (C)	(9,28)(27,W17)		17		25	320	333		0.97		0.34
ZACK (H)	(10,W29)(12,W15)	20		10		248	324	0.32		0.40	
(W)	(2,3)(5,12)					293	326				
OSLU (C)	(9,28)(27,W17)		10		11	277	317		0.70		0.50
SCHU (H)	(9,W30)(W10,13)	10		17		290	301	0.62		0.64	
(W)	(2,9)(W5,±W21)					297	316				
LAO (C)	(2)(W10)		14		35	298	300		1.27		0.79
TONN (H)	(1,2)(12,8)	35		17		260	257	0.43		0.71	
(W)	(2,10)(12)					266	294				
HAYS (C)	(11,W32)(12,W5)		54		21	260	287		1.03		0.26
GRYG (H)	(1,2)(5,W17)	2		2		270	316	0.42		0.48	
(W)	(1)(W10,W15)					271	320				
OSLU (C)	(9,28)(W27,W17)		3		7	292	283		1.01		0.39
LOCK (H)	(3,11)(7,12)	14		10		294	292	0.46		0.50	
(W)	(2,3)(W5,14)					297	294				
ZIEG (C)	(2)(7,±27)		15		12	281	305		0.88		0.54

* day of MLC

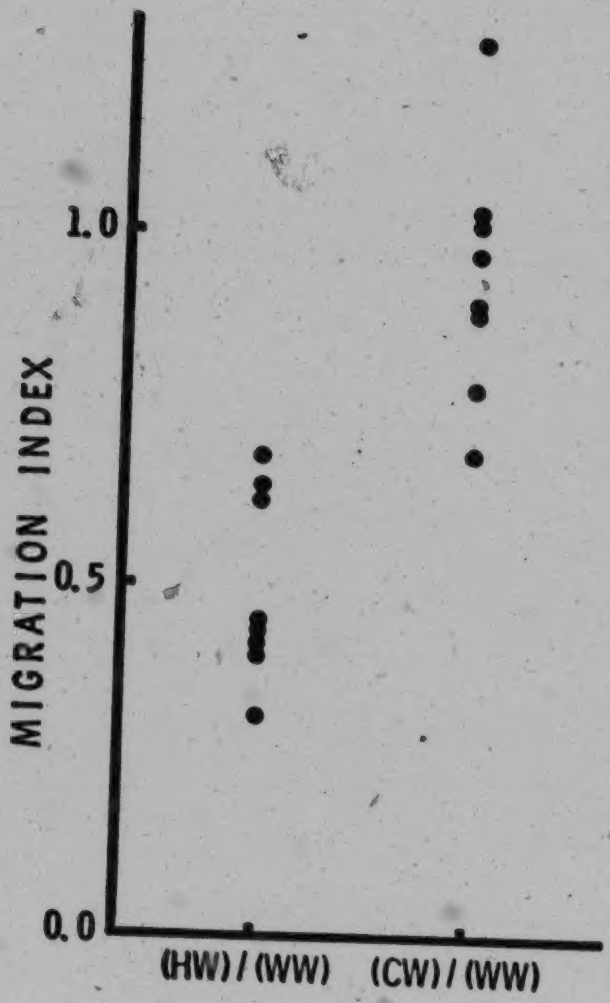

Figure 3 Indirect capillary migration of 5 day MLC of supposedly presensitised individuals (W) with the putative sensitisor (H) compared with an unrelated control (C).

(K) Detection of MIF and Blastogenesis in 5 day MLC between Presensitized and Unsensitized Individuals Using the Indirect Technique

In the putative presensitized combinations, MIF production commenced earlier being detectable by day 3 (Figure 3 and Table XI). In day 5 MLC studies (HW) and (CW) groups again showed normal proliferation as determined by the BI. The mean MI in 8 experiments in the husband and wife (HW) mixed culture supernates was 0.52 ± 0.04 s.e. (Figure 4), whereas that for the control wife-third party supernates (CW) was 0.47 ± 0.05 s.e. The difference is not statistically significant. This allows a conclusion that MIF is produced by 5 day MLC cultures in both normal cultures and in cultures between supposedly sensitized cells and the putative sensitizor cells.

(L) Detection of MIF and Blastogenesis between Unrelated HL-A Identicals

The HL-A antigens of many unrelated individuals were determined by the Tissue Typing Laboratory personnel using the Terasaki microcytotoxicity method (1964). The aim was to determine whether individuals who were identical by typing would produce MIF and blastogenesis after 5 days of MLC. Three unrelated persons had the HL-A profile of (1,2) (8,12). The control (C) was (9,40)(27,17). These three persons were designated (H), (M), and (N) in this section. Three base lines were used for the measurement of MI. The standard MI is defined as the average area of migration in the mixed culture (AB) divided by

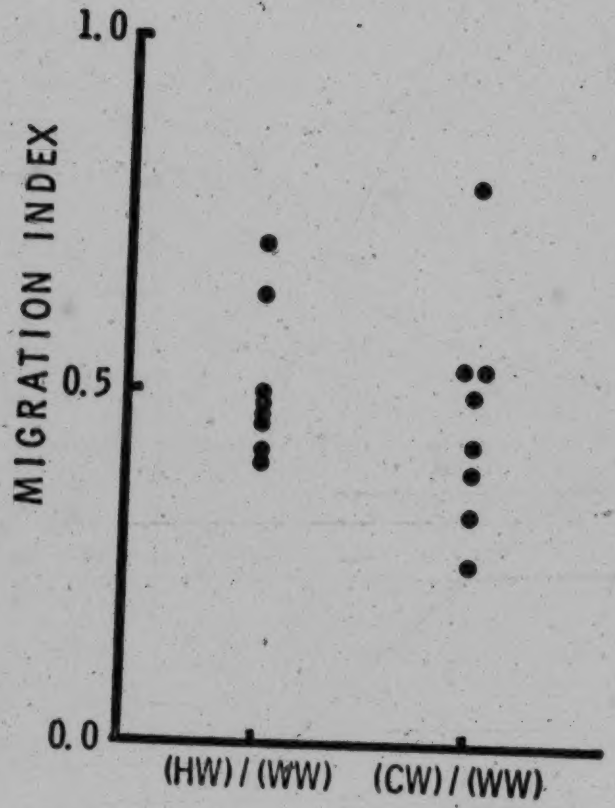

Figure 4 Indirect capillary migration in supernates of 5 day MLC of supposedly sensitised individuals (W) with the putative sensitisor (H) compared with an unrelated control (C).

the average areas of migration in the unmixed culture supernates (AA). In the experiment $(\frac{AB}{AA})$ is the standard MI, but MI defined as $\frac{AB}{BB}$ and $\frac{AB}{\frac{1}{2}(AA + BB)}$ were also introduced to see if there were any significant differences between the three expressions. BI as always were defined as $\frac{AB}{\frac{1}{2}(AA + BB)}$ and the BI among the 3 unrelated HL-A identicals were high. MIF was also detectable in all cultures between HL-A identicals as well as nonidenticals. There is no significant difference between the different expression for MI in this experiment (Table XII). It is concluded that unrelated HL-A identicals not only stimulated each other into blastogenesis in MLC but produced MIF in supernates of 5 day cultures, as do cells that have dissimilar tissue typing. HL-A identical siblings with nonstimulation in MLC (BI < 2.0) were not examined for 5 day supernatant MIF activity.

(M) Characterization of MIF in the MLC Supernates

At least 4 capillary tubes were used to test the effect of each supernate on migration. In a series of 59 experiments, the area of migration of leukocytes from any individual capillary tube did not differ more than ±6% from the average migration area of the 4 capillary tubes. In the inhibitory supernatants, migration inhibition was not due to increased mortality of migrating cells. The viability of the migratory cells (determined by 0.05% trypan blue) at the end of the migration period ranged between 50 and 80%, being somewhat variable from experiment to experiment. In a given experiment, there was no

Table XII

MI (Determined by the Indirect Capillary Migration)
and BI Between Unrelated HL-A Identical and
Nonidenticals* in 5 day MLC

	DIFFERENT EXPRESSION FOR MI			BI
	$\frac{AB}{AA}$	$\frac{AB}{BB}$	$\frac{AB}{\frac{1}{2}(AA+BB)}$	
HC	0.68 (59/86)	0.40 (59/146)	0.51 (59/116)	45
MC	0.59 (51/86)	0.47 (51/109)	0.52 (51/97)	29
NC	0.67 (57/86)	0.57 (57/99)	0.55 (57/92)	30
HM	0.40 (58/146)	0.53 (58/109)	0.46 (58/127)	15
HN	0.45 (66/146)	0.66 (66/99)	0.51 (66/122)	23
MN	0.50 (54/109)	0.54 (54/99)	0.52 (54/104)	24

---

* Individuals H, M, and N were unrelated HL-A identical (1,2)(8,11)
C was a nonidentical control individual (28,W32)(14,W20)

Table XIII

Viability* of Migration Cells (Determined by 0.05% Trypan Blue) after 18 hours of Incubation in Different Supernates

SUPERNATES EXPT.	HW	CW	WW
CLARK	$77[\frac{(177-39)}{177}\times 100\%]$	$70[\frac{(177-52)}{177}\times 100\%]$	$72[\frac{(177-49)}{177}\times 100\%]$
SMITH	$61[\frac{(109-42)}{109}\times 100\%]$	$66[\frac{(109-36)}{109}\times 100\%]$	$56[\frac{(109-48)}{109}\times 100\%]$
TONN	$66[\frac{(216-73)}{216}\times 100\%]$	$63[\frac{(216-80)}{216}\times 100\%]$	$72[\frac{(216-59)}{216}\times 100\%]$
LOCKWOOD	$66[\frac{(115-39)}{115}\times 100\%]$	$78[\frac{(115-25)}{115}\times 100\%]$	$74[\frac{(115-29)}{115}\times 100\%]$
GRAHAM	$51[\frac{(96-47)}{96}\times 100\%]$	$58[\frac{(96-40)}{96}\times 100\%]$	$67[\frac{(96-31)}{96}\times 100\%]$

$$^{*}\text{ Viability} = \frac{\text{total cells per area} - \text{stained cells per area}}{\text{total cells per area}} \times 100\%$$

significant difference in the viability of cells in the control, unmixed cell, supernatants compared with those in the experimental, mixed culture, supernatants (Table XIII).

The MIF activity was unaffected by heating at 56°C for 30 minutes, but was extremely cold labile. Unheated, the MIF of five day MLC supernatants was 0.35 ± 0.08 s.e., after heat treatment, the mean activity was 0.43 ± 0.13 s.e. (not significantly different). If the supernatants were kept at -70°C for 72 hours or more, the MI of the freshly thawed supernatants was 1.07 ± 0.10 s.e. which was significantly different from the other two MI (Table XIV). It is concluded that supernates have activity in migration inhibition which is consistent with the known effects of thermal change on MIF and that failure to migrate is not due to cell death.

(N) Demonstration of Migration-Inhibition after 6 hours Incubation of Cell Mixtures

Peripheral blood lymphocytes were harvested by the Ficoll Isopaque technique and the cells were cultured at the concentration of 2×10^6 cell per ml in TC 199 medium with 100 unit/ml of penicillin and 100 μg/ml of streptomycin. After 6 hours of incubation the cells were resuspended in the supernates and packed into capillary tubes.

The tubes were cut at the cell fluid interphase and allowed to migrate in the respective culture supernates. After 18 hours of

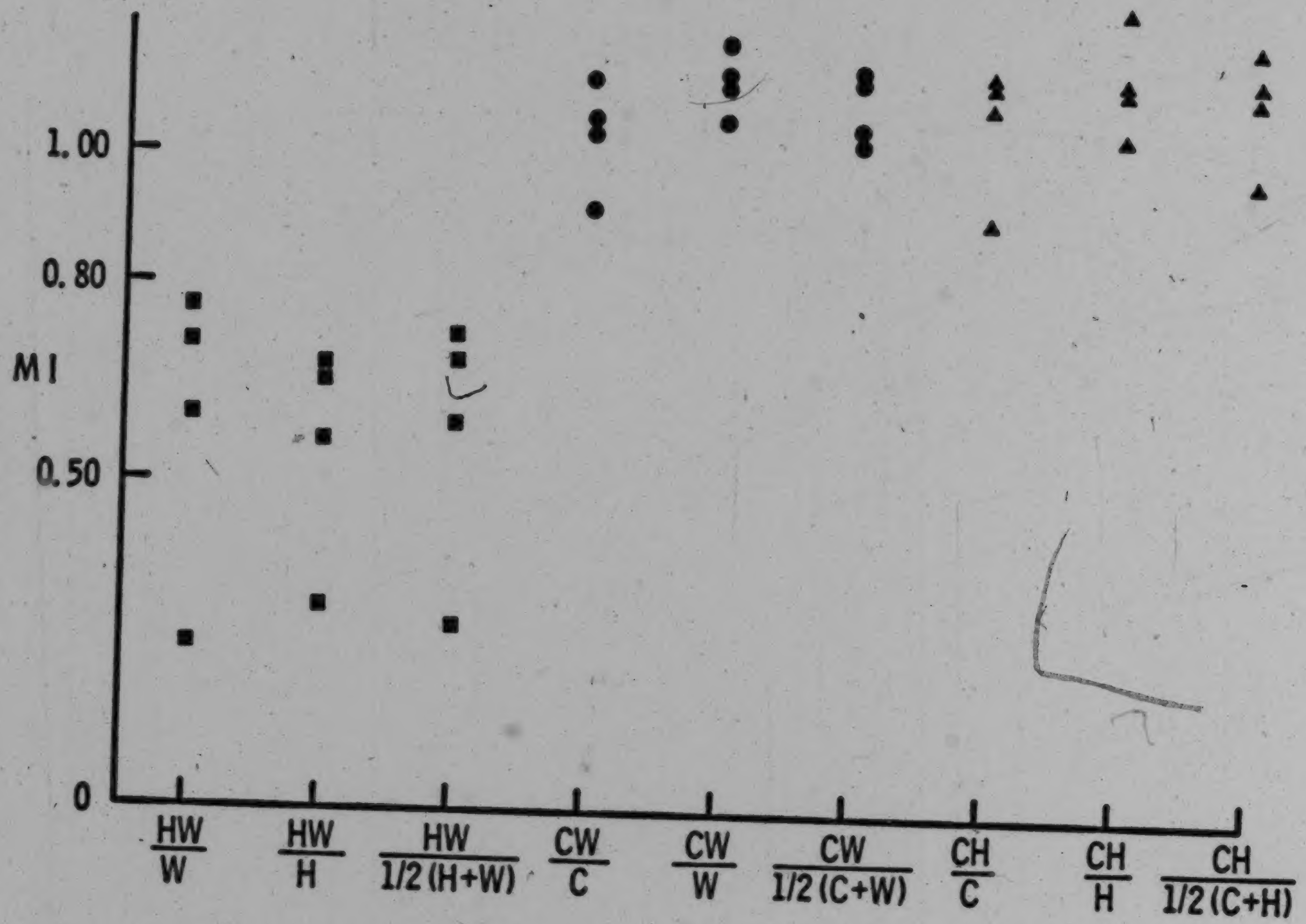

Figure 5 Comparison of MI of direct capillary migration determined by different expressions.

Table XIV

MI of HW Supernates after Heat and Cold Treatment

EXPT.	NORMAL	HEATED*	FROZEN**
A	0.51 (49/88)	0.66 (58/73)	1.16 (91/79)
B	0.26 (11/43)	0.36 (12/32)	1.15 (66/50)
C	0.18 (4/21)	0.18 (5/29)	0.90 (17/19)
D	0.59 (68/116)	0.73 (70/97)	1.22 (139/114)
E	0.21 (18/86)	0.22 (21/99)	0.92 (71/78)
MEAN MI	0.35±0.08 s.e.	0.43±0.13 s.e.	1.07±0.06 s.e.

* Heated = 56°C for 30 minutes

** Frozen = - 72°C for ≥ 72 hours

incubation at 37°C, the MI was determined. In 21 HW combinations 20 had MI less than 0.80 (except experiment 2557). Two out of 21 CW cultures also had MI less than 0.80 (experiments 2675 and 2771) (Table XV)(Figure 6). In all these cases the multiparous women had had antibodies against their husbands' histocompatibility antigen immediately after delivery and also approximately 6 weeks later. In 3 of 5 instances when the wives had antibodies detectable after delivery, but none when the experiments were done, migration-inhibition was found in the HW cell combination (Table XVI)(Figure 7). 4 of 8 multiparous women who had 5 or more children without detectable antibodies against histocompatibility antigens showed migration inhibition in HW combination (Table XVII)(Figure 8). It is concluded that when the wife has cytotoxic antibodies against her husband's cells direct HW studies nearly always show MIF activity. The failure of HW incubate mixtures in direct migration, is quite often seen even when a supposedly sensitized woman is negative by the humoral lymphocytotoxicity test. Thus CMI is induced by gravidity and persists longer than cytotoxic antibodies in the postpartum period.

(O) Detection of Blocking Factor that Prevents Cell Migration - Inhibition in the Direct Capillary Migration and the Direct Agarose Migration Techniques

Autologous serum was obtained from the multiparous women and was added in 1.5% into each combination of cultures. In 7 experiments

Table XV

Direct Migration of Presensitized Individuals with Cytotoxic Antibodies

EXPT.	MI		HL-A ANTIGENS		
	$\frac{HW}{W}$	$\frac{CW}{W}$	H	W	C
2479	0.35 (40/120)	0.85 (107/126)	(3,11) (7,12)	(2,3) (W5,14)	(2) (7,27)
2550	0.43 (67/153)	0.96 (146/153)	(11,28) (14,W18)	(2,3) (7,27)	(2) (7,27)
2547	0.40 (60/148)	0.86 (119/148)	(2,3) (W5)	(1,28) (7,W10)	(1,9) (17)
2557	1.11 (151/136)	1.16 (157/136)	(11,9) (7,5)	(2,9) (7,W10)	(2,10) (W22,14)
2561	0.74 (82/111)	0.97 (108/111)	(2,10) (7,W22)	(1,3) (8,7)	(1,9) (17)
G 76	0.45 (36/80)	1.04 (83/80)	(1,W30) (,W18)	(2,10) (12W5)	(2,3) (7,12)
2563	0.64 (58/91)	1.11 (101/91)	(2) (13,W10)	(11,W29) (12)	(1,11) (7,8)
2568	0.60 (44/73)	1.14 (106/73)	(2) (12,17)	(3,11) (7±27)	(1,11) (7,8)
2575	0.33 (47/142)	0.77 (110/142)	(3,W30) (5,W18)	(2,3) (7,27)	(28,W32) (W22,14)
2685	0.61 (57/94)	0.93 (87/94)	(10,W29) (12,W15)	(2±28) (5,W18)	(1,W31) (8,W10)
2714	0.40 (41/107)	0.88 (94/107)	(1,2) (7,W15)	(2,3) (W5)	(1,11) (7,8)

Table XV

EXPT.	MI		HL-A ANTIGENS		
	$\frac{HW}{W}$	$\frac{CW}{W}$	H	W	C
2741	0.48 (81/112)	1.04 (118/117)	(1,2) (8,W10)	(3,10) (5,W5)	(1,11) (7,8)
2748	0.79 (76/96)	1.00 (98/96)	(1,3) (7,8)	(1,2) (14,8)	(1,W30) (8,W10)
G203	0.62 (70/112)	0.90 (100/112)	(2,10) (W22)	(17,12)	(1,11) (7,8)
G233	0.72 (26/36)	0.97 (35/36)	(W5,17)	(10±W29) (27,W10)	(1,W30) (8,W10)
2771	0.74 (38/51)	0.65 (33/51)	(1,2)	(3,11) (±5,7)	(2) (W10)
2752	0.63 (58/96)	0.88 (88/92)	(2,3) (27,W10)	(10,11) (W5)	(2±32) (12)
2740	0.60 (60/100)	0.86 (103/120)	(2±28) (12,W5)	(2) (W21,12)	(2,3) (27,W10)
2782	0.78 (t1/66)	1.02 (67/66)	(2,9) (12,W22)	(2) (7)	(28,W32) (W22,14)
2805	0.71 (76/108)	0.85 (92/108)	(3,28) (14,W18)	(1,9) (8,7)	(2) (7,27)
2809	0.62 (78/126)	1.04 (131/120)	(3,10) (7,W18)	(2,W32) (7,W5)	(2,W21) (12,13)

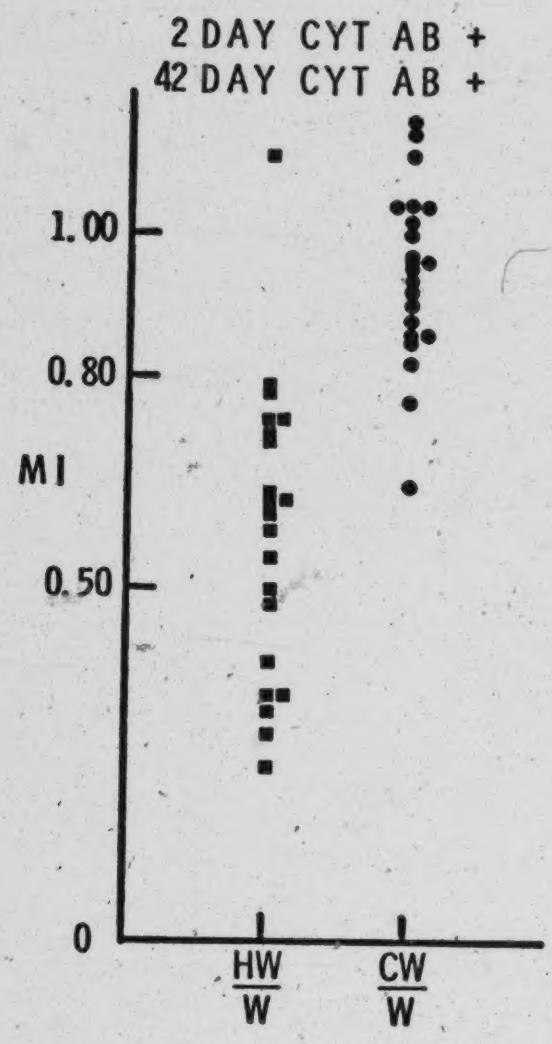

Figure 6 Comparison of direct capillary migration inhibition between individuals with cytotoxic antibodies and unrelated individuals against the putative sensitisor.

Table XVI

Direct Migration of Presensitised Individuals whose Cytotoxic Antibodies have Disappeared 6 weeks after Delivery

EXPT.	MI		HL-A ANTIGEN		
	$\frac{HW}{W}$	$\frac{CW}{W}$	H	W	C
G146	1.12 (122/109)	0.89 (96/109)	(2) (W5,W22)	(28,W29) (12,14)	(3,11) (7,12)
2547	0.64 (127/195)	1.03 (201/195)	(2,3) (W5)	(1,28) (7,W10)	(1,11) (7,8)
2547	0.72 (109/152)	1.10 (154/152)	(2,3) (W5)	(1,28) (7,W10)	(1,11) (7,8)
2741	0.71 (64/90)	1.22 (110/90)	(1,2) (7,W10)	(3,10) (5,W5)	(3,9) (5)
2801	0.59 (88/164)	1.14 (187/164)	(3,W30) (27,W18)	(2) (W15,W10)	(2,9) (7,W15)
G189	1.02 (78/77)	1.07 (83/77)	ND*	ND	ND
2741	0.48 (46/95)	1.03 (108/95)	(1,2) (8,W5)	(3,10) (5,W10)	(3,9)

*ND = Not done

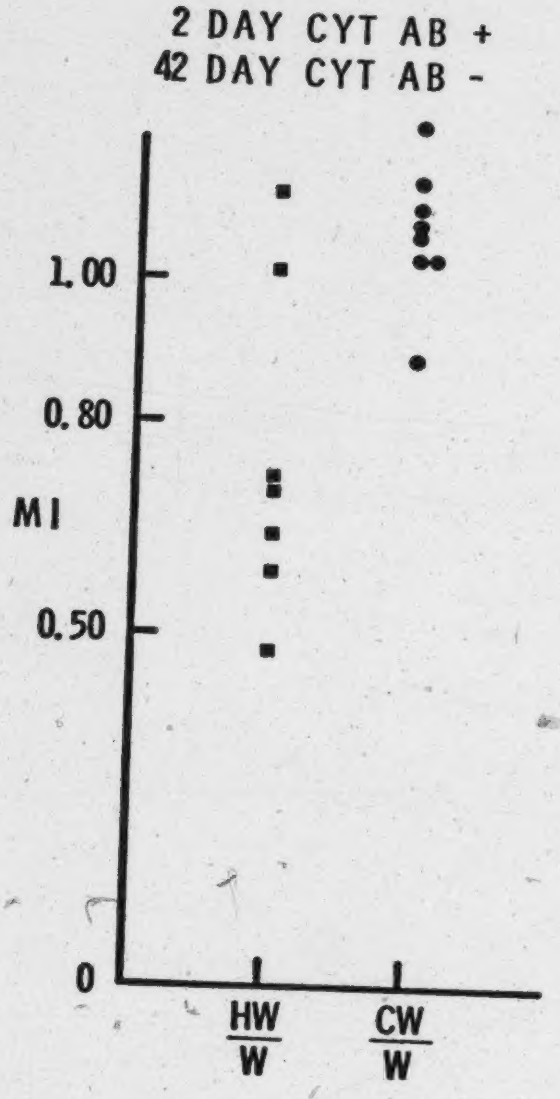

Figure 7 Comparison of direct capillary migration inhibition between individuals with disappeared cytotoxic antibodies and unrelated individuals against the putative sensitisor.

Table XVII

Direct Migration of Presensitized Individuals Without Cytotoxic Antibodies

EXPT.	MI		HL-A		
	$\frac{HW}{W}$	$\frac{CW}{W}$	H	W	C
2548	1.22 (96.78)	1.07 (84/78)	(3.9) (7±17)	(2±28) (17)	(1,11) (7.8)
2611	0.78 (59/76)	1.14 (104/70)	(3,W29) (12,W15	(3,10) (7,12)	(11,W32) (12,W5)
2624	0.71 (57/80)	1.04 (109/80)	(3) (7,12)	(2,11) (8,W10)	ND*
2619	0.77 (70/91)	1.00 (91/91)	(2,9) (7,27)	(2,W32) (12,W10)	ND*
2631	1.02 (107/105)	0.96 (101/105)	(2,9) (12,13)	(10) (8,12)	(2) (7,27)
2563	0.64 (54/84)	1.11 (93/84)	(2) (W10,13)	(11,W29) (12)	(1,11) (7,8)
2725	1.13 (147/130	1.06 (138/130)	ND	ND	ND
2661	1.01 (113/112)	1.07 (120/112)	ND	ND	ND

*ND = Not done

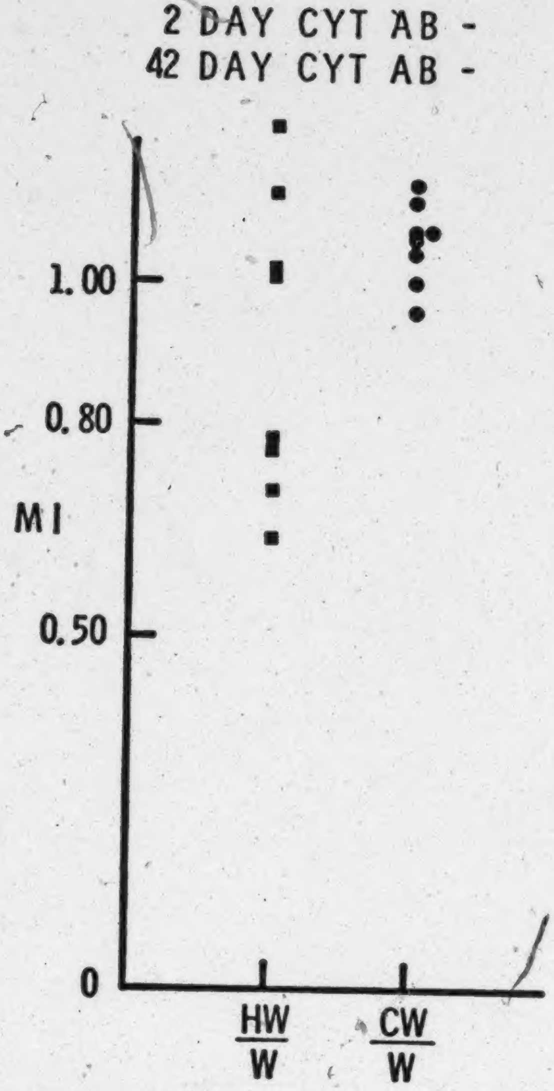

Figure 8 Comparison of direct migration inhibition between individuals without cytotoxic antibodies and unrelated individuals against the putative sensitisor.

the cells of each culture were divided into 2 gourps, one for capillary migration and the other for agarose migration. Six out of 7 experiments demonstrated inhibitions with the capillary migration technique and in all 6 cases there was no migration inhibition when the cultures had wife's autologous serum. In the agarose technique all 7 cases showed migration inhibition, and in all the inhibition was prevented by wife's autologous serum so that the HW mixed cells migrated normally (Table XVIII, Figure 9). It is concluded that the results from the agarose technique are comparable to that of the direct capillary migration and that the wife's serum contains a "factor" that prevents migration-inhibition.

(P) Comparison of Blocking Factor in Migration Inhibition and UMLC

13 couples in which the wife had cytotoxic antibodies at the time of delivery, were chosen for comparison of MIF blocking factor and UMLC inhibition. In 1 case the wife had no cytotoxic antibodies at the time of experiments; in this instance there was no inhibition of migration even in the absence of autologous serum so the possible blocking effect could not be examined. In the other 12 cases, migration of HW mixed cells was inhibited (Table XIX), and blocking factor was present in 10 cases. It is concluded that there is significant similarity between a serum that inhibits MIF activity and one that inhibits the wife's cell response when stimulated in UMLC by the husband's cells.

Table XVIII

Comparison of Direct Capillary and Agarose Techniques

EXPT.	HL-A PROFILE			M.I.							
				CAPILLARY				AGAROSE			
	H	W	C	$\bar{s}$ WS		$\bar{c}$ WS		$\bar{s}$ WS		$\bar{c}$ WS	
				HW/W	CW/W	HW/W	CW/W	HW/W	CW/W	HW/W	CW/W
G233	(W5)	(10±W29) (27,W10)	(1,W30) (8,W10)	0.72 (26/36)	0.92 (33/36)	1.06 (44/42)	1.12 (43/42)	0.46 (34/75)	0.96 (72/75)	1.09 (89/81)	1.20 (97/81)
2752	(2,3) (27,W10)	(10,11) (W5)	(2±W32) (12)	0.63 (58/93)	0.96 (89/93)	0.97 (80/82)	1.10 (91/82)	0.29 (18/63)	0.82 (52/63)	1.11 (93/84)	1.18 (99/84)
2740	(2,W32) (12)	(2) (W5,12)	(2,3) (27,W10)	0.50 (60/120)	0.86 (104/120)	1.07 (128/119)	0.77 (93/119)	0.17 (16/92)	1.15 (106/92)	1.13 (128/113)	1.09 (124/113)
2782	(2,9) (12,W22)	(2) (7)	(28,W32) (W22,14)	0.78 (51/66)	1.02 (67/60)	1.16 (95/82)	1.10 (90/82)	0.48 (36/75)	0.99 (74/75	1.15 (102/89)	1.05 (94/89)
2805	(3,28) (14±17)	(1,9) (8,7)	(2) (7,27)	0.71 (76/108)	0.85 (92/108)	0.95 (94/99)	0.91 (90/99)	0.39 (42.41)	0.90 (39/41)	1.07 (58/63)	0.29 (64/65)
2809	(3,10) (7,W18)	(2,W32) (7,W5)	(2,W32) (12,13)	0.62 (78/126)	1.04 (131/126)	1.09 (129/118)	1.02 (121/118)	0.58 (24/41)	0.95 (39/41)	0.92 (58/63)	1.01 (64/65)
2807	(2,9) (7W15)	(1,3) (7,8)	(3W30) (27,W18)	0.89 (63/71)	1.09 (77/71)	1.14 (70/62)	1.00 (63/62)	0.39 (26/66)	1.06 (70/66)	0.82 (73/89)	0.91 (81/89)

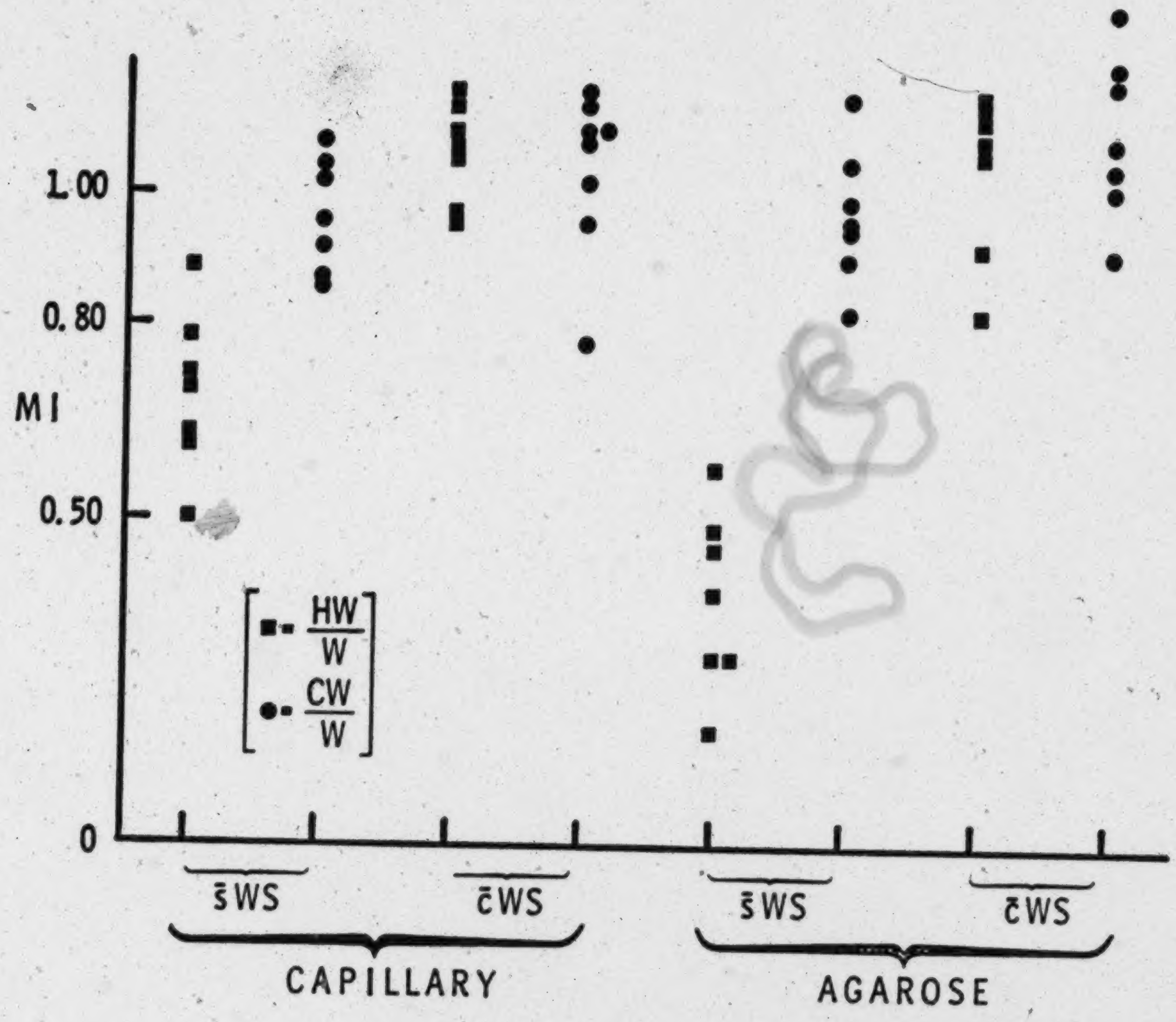

Figure 9 Comparison between direct capillary and agarose migration inhibition

Table XIX

MIF Blocking and UMLC Inhibition

	MIF		UMLC
	HW	HW & WS	HW & WS
G205	-	-	-
2801	+	+[**]	-
2825	+	+	+
G275	+	+	+
2810	+	+	+
2479	+	+	+
2771	+	-	+
2807	+	+	+
2805	+	-	-
2859	+	+	+
2502[*]	+	+	-
2500[*]	+	+	+
2540[*]	+	+	+

[*] Experiments done > 42 days postpartum

[**] Blocking or no inhibition of migration

(Q) Specificity of Blocking Factor

In 6 couples, all the women had demonstrated migration inhibition to the husbands' antigens. And all the HW cells migrated normally in the wife's autologous serum. If an unrelated serum from another multiparous woman substituted the wife's autologous serum, there was still inhibition in the HW cell migration (Table XX). It is concluded from this preliminary specificity study that the blocking factor is a serum factor with specificity to the sensitized or sensitizing individuals though whether this specificity can be defined by the husbands' HL-A antigens has not been determined.

(R) Correlation of 3 *in vitro* Systems to Detect CMI (MIF, CML, and CI) in Multiparous Women Cells Tested Against Panel Cells

In the study of 10 multiparous women who were sensitized only by their husbands' histoincompatibility antigens, their cells were tested against a constant panel of five cells by MIF, CML, and CI (Table XXI). Between MIF and CML 50 tests were done and there were 21 positive tests, 9 of which were positive when both MIF and CML were positive, 7 by MIF above and 5 by CML alone. The chi square shows 9.41 with 1 degree of freedom and the correlation between the two tests is significant ($p < 0.01$)(Table XXIIa). When MIF and CI were compared there were 40 tests done only 3 of which were positive when both MIF and CI were positive, 9 were positive by MIF alone and 5 by CI alone (Table XXIIb). There is no significant correlation between these two

Table XX

Specificities of Blocking Factor

EXPT.	HL-A ANTIGENS			M.I.		AUTOLOGOUS WIFE SERUM	M.I.		ALLOGENEIC WIFE SERUM	M.I.	
	H	W	C	HW	CW		HW	CW		HW	CW
2752	(2,3) (7,W10)	(10,11) (W5)	(2±W32) (12)	0.29 (18/63)	0.82 (52/63)	2752	1.11 (93/84)	1.18 (99/84)	2740	0.73 (68/80)	1.13 (90/80)
2740	(2,W32) (12)	(2) (W5,12)	(2,3) (27,W10)	0.17 (16/92)	1.15 (106/92)	2740	1.13 (128/113)	1.09 (124/113)	2752	0.34 (25/70)	1.02 (77/70)
2807	(2,9) (7,W15)	(1,3) (7,8)	(3,W30) (27,W18)	0.39 (26/66)	1.06 (70/66)	2807	0.82 (73/89)	0.91 (81/89)	2801	0.44 (22/51)	0.96 (48/51)
2801	(3,W30) (27,W18)	(2) (W15,W10)	(2,9) (7,W15)	0.43 (50/117)	0.96 (112/117)	2801	0.88 (82/94)	0.91 (85/94)	2807	0.33 (40/123)	1.06 (130/123)
2825	(2,3) (7,14)	(2,3) (7,W15)	(2,11) (12,W22)	0.56 (44/79)	1.14 (90/79)	2825	1.06 (68/65)	1.11 (72/65)	2810	0.47 (32/69)	1.05 (72/69)
2810	(2,11) (12,W22)	(1,2) (7,8)	(2,3) (W5,14)	0.68 (34/50)	0.98 (49/50)	2810	0.94 (44/47)	0.99 (46/47)	2825	0.73 (28/39)	0.85 (33/39)

Table XXI

Comparison of MIF, CML, and CI by the Cells of
Multiparous Women Tested Against Their Husbands and
a Constant Panel of 5 Cells

EXPT.	WIFE HL-A	HUSBAND HL-A	PANEL** AND HUSBAND CELLS	MIF	CML	CI
LOCK	(2,3)(14,W5)	(3,11)(7,12)	H	+	+	ND*
			1	+	+	ND
			2	+	-	-
			3	+	-	-
			4	-	-	-
			5	-	-	-
OGLU	(±3)(12,W10)	ND	H	ND	ND	ND
			1	+	-	ND
			2	+	+	-
			3	+	+	+
			4	-	+	-
			5	-	+	-
LALI	(1,9)(7,8)	(3,28)(14,12)	H	-	-	ND
			1	-	-	ND
			2	-	-	-
			3	-	-	-
			4	-	-	-
			5	-	-	-
OLYK	(1,W29)(7)	(2)(7)	H	+	-	ND
			1	-	-	ND
			2	+	+	-
			3	+	-	-
			4	-	-	-
			5	-	-	+
GALL	(3)(7,W5)	(1,11)(8)	H	+	+	ND
			1	+	-	ND
			2	+	+	-
			3	+	+	-
			4	-	-	-
			5	-	-	-

Table XXI

EXPT.	WIFE HL-A	HUSBAND HL-A	PANEL** AND HUSBAND CELLS	MIF	CML	CI
WHIT	(3)(7,W5)	(1,11)(8)	H	+	+	ND
			1	+	–	–
			2	+	+	–
			3	+	+	–
			4	–	–	–
			5	–	–	–
DUCL	(2,11)(17,W5)	(9,11)(12)	H	+	+	ND
			1	+	+	ND
			2	+	–	+
			3	+	–	+
			4	–	–	+
			5	–	–	+
KREP	(2)(17)	(3,10)(17,27)	H	–	+	ND
			1	–	–	ND
			2	–	+	–
			3	–	+	–
			4	–	+	–
			5	–	–	–
ZONN	(9,10)(12,18)	(3,28)(5W5)	H	+	–	ND
			1	–	–	–
			2	+	+	–
			3	+	+	–
			4	–	–	–
			5	–	–	–
ZAHA	(2,10)(W10)	(2,10)(12)	H	–	–	ND
			1	–	–	ND
			2	–	–	–
			3	–	–	+
			4	–	–	–
			5	–	–	+

*ND = Not done

**HL-A profile of constant panel cells

1 = (1,W32)(12,W5)
2 = (1,W30)(8,W10)
3 = (1,11)(7,8)
4 = (W32,28)(14,22)
5 = (10,W32)(17±W16)

Table XXIIa

Comparison of MIF and CML in the Multiparous
Women Study by the Chi Square Test

		MIF	
		+	-
CML	+	9	5
	-	7	29

$x^2 = 7.37$

$p < 0.01$

Table XXIIb

Comparison of MIF and CI in the Multiparous
Women Study by the Chi Square Test

		MIF	
		+	-
CI	+	3	5
	-	0	23

$x^2 = 0.007$

p = Not significant

Table XXIIc

Comparison of CML and CI in the Multiparous
Women Study by the Chi Square Test

		CML	
		+	-
CI	+	1	7
	-	11	21

$x^2 = 0.06$

p = Not significant

assay systems. Between CML and CI there were 19 positive tests out of 40 but only in 1 case were both CML and CI positive. CML was the only positive test in 11 cases and CI in 7 cases (Table XXIIc). There is no correlation between CML and CI. When all 3 *in vitro* assays were compared there were 9 instances where 2 or more of the 3 assays were positive MIF was always positive (Figure 10). Of the 15 instances when only 1 test was positive MIF was the single positive test in 3 occasions.

(S) Correlation Between MIF and the HL-A Disparities Among Wife, Husband, and Panel Test Cells

In the 10 multiparous women study, cytotoxic antibodies from 7 multiparous women developed against the husbands' antigens as well as against the panel of 5 cells. The sera of 5 of these women were further tested against a panel of 100 cells to determined HL-A specificity. Table XXIII shows a comparison of the HL-A specificities determined by a panel of 5 cells and one of 100 cells, and the HL-A specificities determined by a panel of 100 cells are also the HL-A specificities determined by a panel of 5 cells except in the case of LOCK in which there was an anti 27 antibodies not detectable by the panel of 5 cells however, HL-A 27 crossreacts strongly with HL-A 7 which the husband had. It is concluded that the cytotoxic antibodies that develop against a panel of 5 cells are explicable in terms of HL-A disparity of the husband towards the wife.

Table XXIII*

HL-A Specificities of Multiparous Women Determined by a Panel of 5 Cells and 100 Cells

EXPT.	HL-A SPECIFICIES	
	5 CELL PANEL	100 CELL PANEL
LOCK	12	12, 27
LALI	3, 14	14
GALI	1, 8, W10	1
OGLU	7	7
DUCL	9, 12	9

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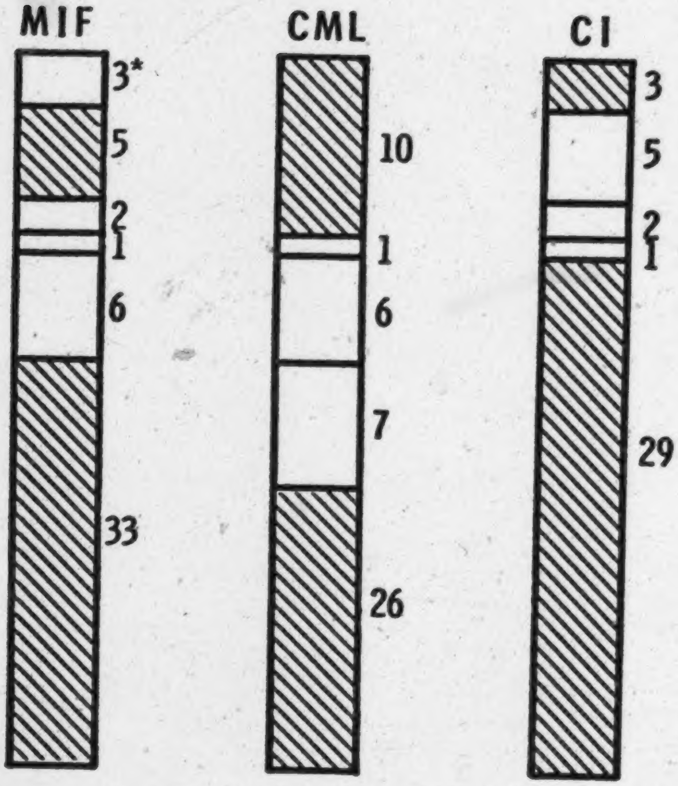
*Data supplied by the Tissue Typing Laboaratory, University of Alberta Hospital, Edmonton, Canada.



☐ POSITIVE REACTION

▨ NEGATIVE REACTION

* Figures denote number of tests

Figure 10 Comparison of MIF, CML, and CI in the multiparous women study

In the MIF experiment which was done in this case 4-17 month postpartum (average of 11 month after delivery), migration-inhibition activity was demonstrable in 6 couples. Among the panel cells that also reacted positively with the wife's cells, 6 tests may be due to the fact that the panel cells shared HL-A antigen with the husband; in 4 tests the panel cells possessed HL-A cross reacting antigen with the husbands HL-A antigens; 3 positive tests were not explicable by the HL-A system. It is concluded that the cytotoxic antibodies against a panel of 5 cells are directly correspondent to the HL-A system but the migration-inhibition phenomenon is not entirely due to HL-A disparity between sensitizing and sensitized cells.

(T) Correlation of 3 *in vitro* Systems (MIF, CML, and CI) to Detect CMI in Haemodialysis Patients' Cells Tested Against Panel Cells

In the study of 12 haemodialysis patients who had been multitransfused, their cells were tests against a constant panel of 5 cells by MIF, CML, and CI (Table XXIV), although it is impossible to determine which HL-A antigens were present on the cells that were responsible for sensitization. There were 56 tests in which both MIF and CML were done. Of the 28 positive tests 15 were positive by both MIF and CML 4 by MIF alone and 9 by CML alone. The chi square shows a value of 13.15 with 1 degree of freedom and the correlation between the tests is significant (Table XXVa). However there is no significant correlation between MIF and CI as well as CML and CI (Table XXVb and

Table XXIV

Comparison of Cytotoxic Antibodies, MIF, CML and CI
in the Dialysis Patients Study Against a Constant Panel of 5 Cells

EXPT.	PANEL CELLS	MIF	CML	CI	CYT
RAWL	1	+	+	−	+
	2	−	−	−	−
	3	+	+	−	+
	4	−	+	−	+
	5	+	−	−	+
SCHM	1	−	−	−	+
	2	−	+	−	+
	3	−	+	−	+
	4	+	+	−	+
	5	+	+	+	+
COLS	1	−	−	−	−
	2	−	+	−	−
	3	−	−	−	−
	4	−	−	−	−
	5	+	+	−	−
JOLS	1	−	−	−	−
	2	−	+	−	−
	3	+	−	−	+
	4	−	−	−	−
	5	−	+	−	−
AOLS	1	+	ND	−	+
	2	−	+	−	+
	3	+	+	−	−
	4	+	+	−	−
	5	−	−	−	−
SLIN	1	+	+	+	+
	2	+	+	+	+
	3	+	+	−	+
	4	+	+	+	+
	5	−	+	+	+

Table XXIV

EXPT.	PANEL CELLS	MIF	CML	CI	CYT
DSCH	1	-	-	-	-
	2	+	-	+	-
	3	+	+	-	-
	4	+	+	-	-
	5	+	+	-	-
FARQ	1	+	-	+	+
	2	-	-	+	+
	3	-	-	-	-
	4	-	+	-	+
	5	ND	ND	-	+
BABL	1	+	+	+	+
	2	-	-	+	+
	3	-	-	-	+
	4	+	+	-	-
	5	ND	ND	ND	-
VANE	1	-	-	-	-
	2	-	-	+	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-
CARS	1	-	-	-	-
	2	-	-	-	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-
SHEW	1	-	-	-	-
	2	-	-	+	-
	3	-	-	-	-
	4	-	-	-	-
	5	-	-	-	-

Table XXVa

Comparison of MIF and CML in the Dialysis Patients Study

		MIF	
		+	-
CML	+	15	9
	-	4	28

$x^2 = 13.15$

$p < 0.01$

Table XXVb

Comparison of MIF and CI in the Dialysis Patients Study

		MIF	
		+	-
CI	+	7	7
	-	13	31

$x^2 = 1.17$

p = Not significant

Table XXVc

Comparison of CML and CI in the Dialysis Patients Study

		CML	
		+	-
CI	+	6	8
	-	18	24

$x^2 = 0.09$

p = Not significant

Table XXVIa

Comparison of Cyt. ab. and MIF in the Dialysis Patients Study

		MIF	
		+	-
CYT AB	+	13	10
	-	7	28

$x^2 = 6.66$

$p < 0.01$

Table XXVIb

Comparison of Cyt. ab. and CML in the Dialysis Patients Study

		CML	
		+	-
CYT AB	+	15	7
	-	9	25

$x^2 = 7.87$

$p < 0.01$

Table XXVIc

Comparison of Cyt. ab. and CI in the Dialysis Patients Study

		CI	
		+	-
CYT AB	+	9	14
	-	4	31

$x^2 = 4.6$

p = Not significant

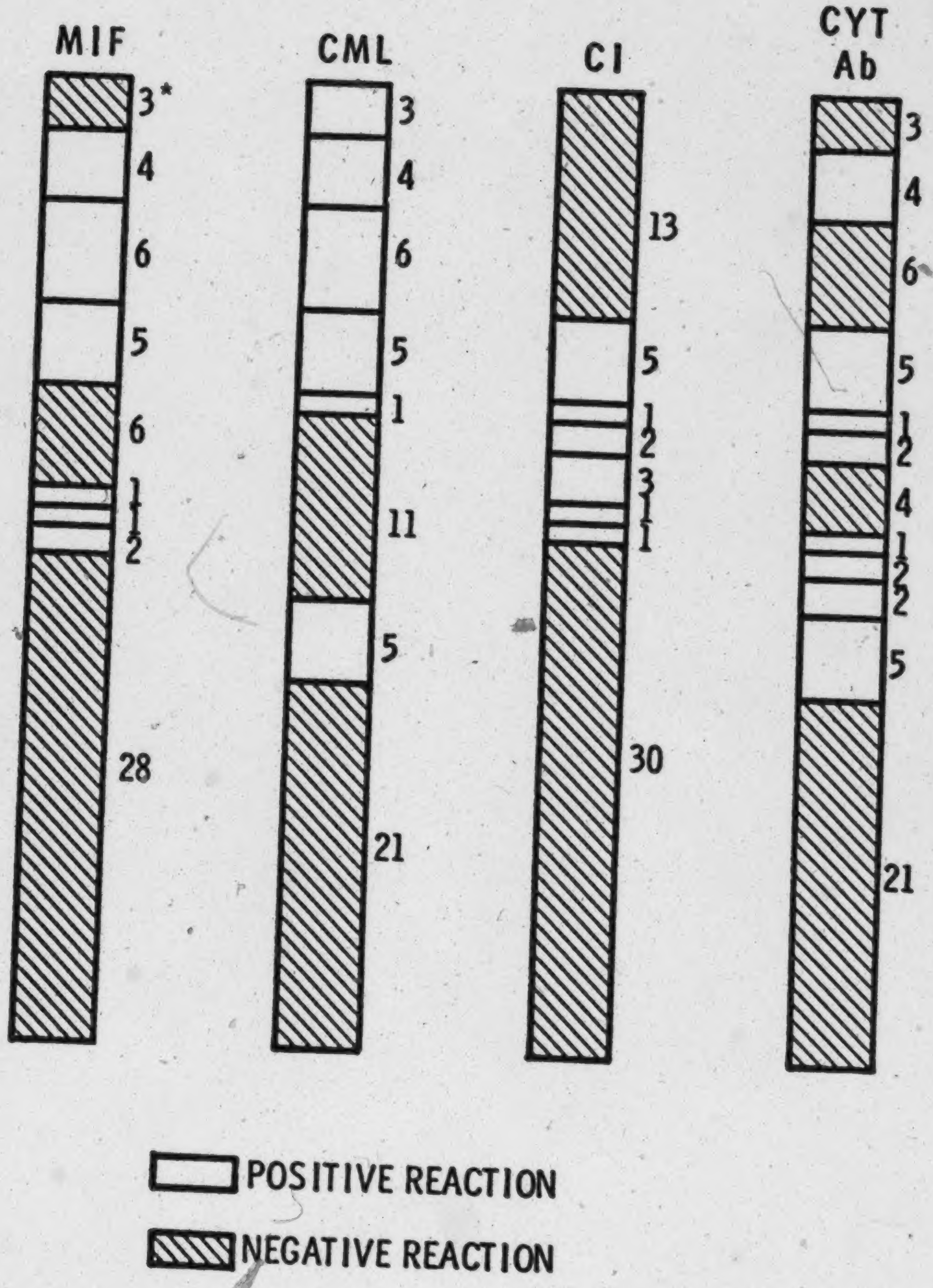
XXVc). When MIF and CI were compared there were 27 positive tests, 7 of which were positive for both MIF and CI (Table XXIV). 26 out of the 32 positive tests for CML and CI showed discrepancy (Table XXIV).

When all the 3 *in vitro* assays were compared there were 18 reactions where 2 or more of the 3 systems were positive, MIF was positive in 17 instances (Figure 11). Of the 15 instances when only 1 test was positive MIF was the single positive test in only 2 occasions.

It is concluded that CI does not appear to mark the same specificities as CML and MIF.

(U) Correlation Between MIF and the HL-A Specificity

In the multitransfused patients the sensitizing HL-A antigens could not be determined but the patients sera were tested against the same 5 cell panel to detect cytotoxic antobodies which usually correlate with HL-A specificities. Of the 12 patients studied 7 developed cytotoxic antibodies against one or more panel cells, and 9 had MIF activity against the same panel. There were 58 tests done by both MIF and cytotoxic antibody assays, and 41 tests were not discrepant. The correlation between the two systems by chi square is significant ($p < 0.01$). There is also significant correlation between CML and cytotoxic antibodies ($p < 0.01$) but not between CI and cytotoxic antibodies (Table XXVIa, b, and c).



* Figures denote number of tests

Figure 11 Comparison of cytotoxic antibodies, MIF, CML, and CI in the dialysis patients study

(V) Effects of Rabbit Anti Human Gamma Globulin in Culture

Rabbit anti-serum to human gamma globulin was obtained in lyopholized form (Miles, Pentex Research Products) and dissolved in 2 cc of distilled water. To each million cells 1 µl, 10 µl or 100 µl of the serum was added. Cultures were set up in parallel with cultures containing ABS only. The migration index was low in HW incubates and normal in CW incubates under various concentration of rabbit anti human immunoglobulin (Figure 12). From this it is concluded that migration-inhibition, in direct migration of 6 hour HW incubates, is not due to antibodies secreted during the incubation and the subsequent migration periods. This provided indirect evidence that MIF is the cause of inhibition in this direct migration system.

(W) Detection of MIF activity after Sephadex G 100 Separation

Supernates from different cultures such as HW, CW, and W that contained ABS and ABS with autologous serum of the W were concentrated 5X, dialysed and passed through a Sephadex G 100 column. The fractions were pooled as G1, G2, G3, G4 and G5 according to the peaks from the charts of the recorder. After lyophilization and concentration of the supernates, guinea pig peritoneal exudate cells (PEC) were allowed to migrate in them (Figure 13) (Table XXVII). MIF activity was detectable only in the HW culture supernates of G-4 and G-5 fraction incubated in the absence of the wife's serum. It is concluded that MIF activity

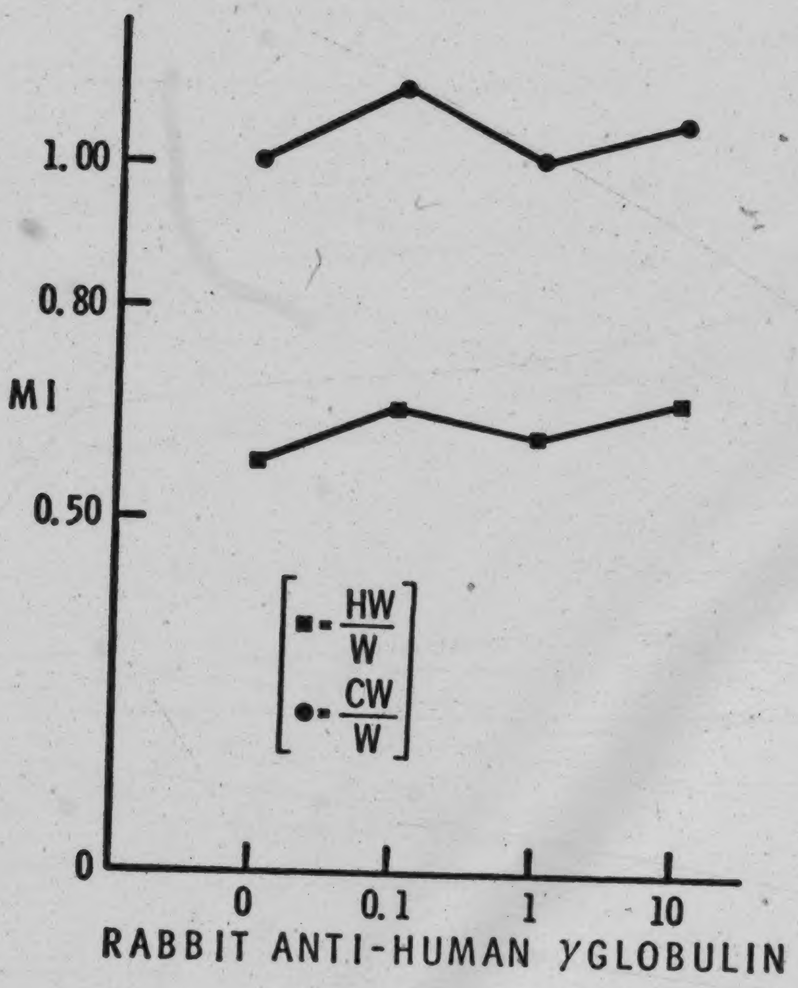


Figure 12 Migration inhibition in the presence of rabbit anti-human gammaglobulin

Table XXVII

Detection of MIF Activity in MLC Supernates After Column Fractionation

	GI	GII	GIII	GIV	GV
Guinea Pig PEC					
ABS					
HW	-	-	-	+	+
W	-	-	-	-	-
CW	-	-	-	-	-
ABS & WS					
HW	-	-	-	-	-
W	-	-	-	-	-
CW	-	-	-	-	-

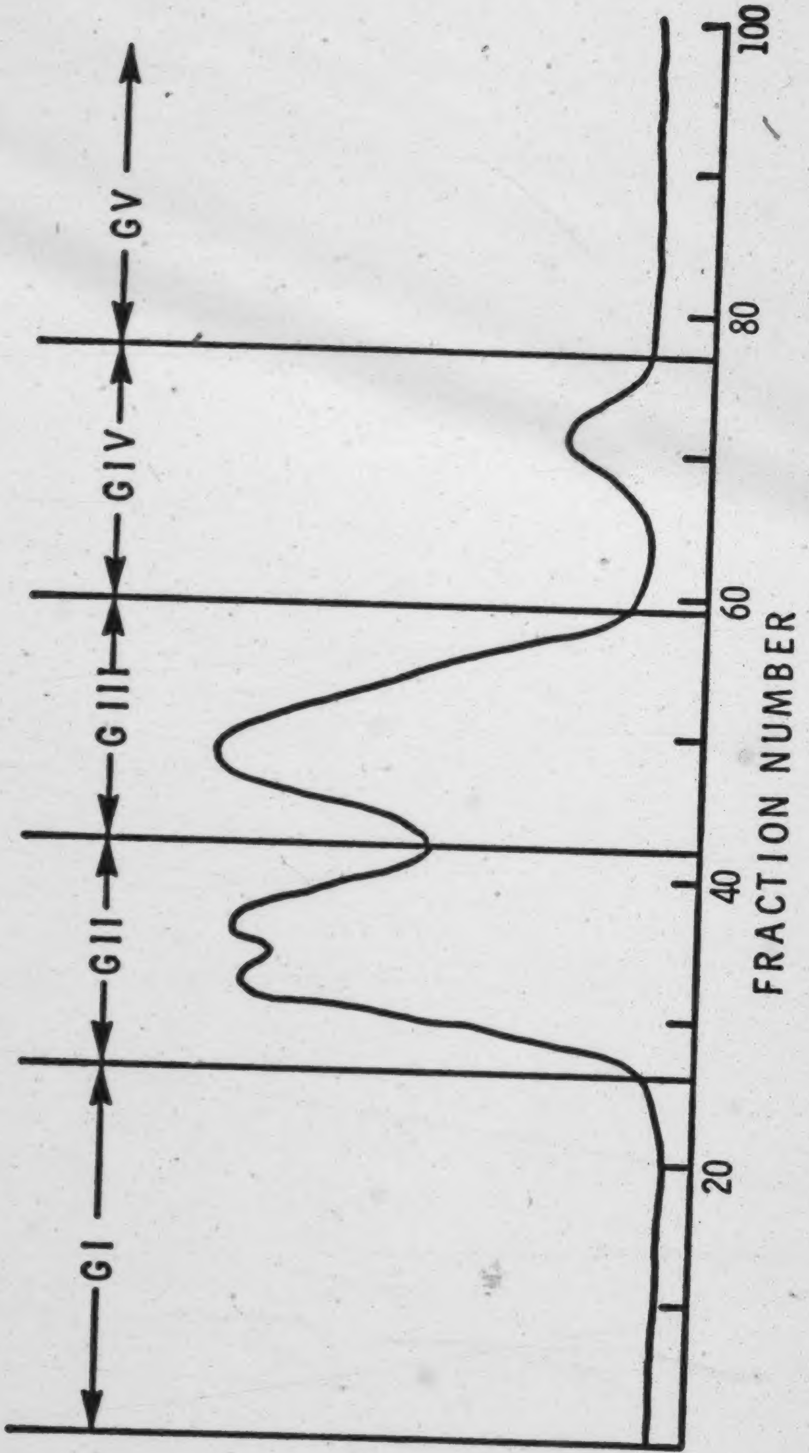


Figure 13 Fractions of MLC supernates after Sephadex G100 separation

is demonstrable by molecules of 70,000 to 23,000 daltons, and autologous serum does not inhibit the activity of MIF but prevents MIF release.

V. DISCUSSION

Studies have established the existence of two distinct populations of immunocompetent cells (Craddock *et al*, 1971). One of these consists of cells (B) capable of producing specific antibodies. The second population is thymus dependent (T) and is responsible for cell mediated immunity. Cells from this population do not secrete immunoglobulins and when stimulated *in vitro* by antigen these cells release substances which have been described as possible effector molecules of cellular immunity (Granger & Kolb, 1968, Bloom & Bennett, 1966, Kasakura & Lowenstein, 1965, Valentine & Lawrence, 1969). MIF is one of these effector molecules.

In vitro lymphocytes from two non HL-A identical individuals would be expected to become sensitized to each other in MLC. As a result lymphocytes after 5 days of culture might not only proliferate but also produce MIF. In 16 experiments supernates from MLC and unmixed lymphocyte cultures were assayed for MIF activity by allowing an unrelated population of human peripheral leukocytes to migrate in them. This procedure is called the indirect migration technique. 5 day MLC between normal unsensitized cells produce MIF whether cultured in human AB serum or FCS (Table II and III). In the case of UMLC where one of the mixed populationshad been irradiated by 7000 R there is also MIF activity detectable in the supernates after 5 day culture (Table IV).

It was shown that the source of the indicator leukocytes is unimportant in the detection of MIF. Cells from either the 3rd party, responder or stimulator were used as migratory cell populations and had MI of 0.38, 0.40, and 0.40 respectively in the same cultured supernates (Table IV). Although the presence of cytophilic antibodies in a 5 day culture cannot be ruled out, the inhibition phenomenon is definitely not specific. MIF is known not to inhibit cell migration specifically (David, 1972). The MI from the 5 day UMLC is not significantly different from the MI from 5 day MLC (Table II, III, and IV) although in the latter case lymphocytes from both individuals can produce MIF. The amount of MIF produced by one population or two is not demonstrable by the indirect migration inhibition technique.

In 5 experiments in which lymphocytes of 2 unrelated individual are set up for 5 day MLC and UMLC, and the cultured cells are used to migrate in the concentrated medium, the average MI are 0.36 and 0.42 respectively (Table V). This is the 5 day direct migration technique. When Rich and Lewis (1932) first described the migration-inhibition phenomenon, they mentioned that lymphocytes could not be inhibited very well. Since then George and Vaughan (1962) have used guinea pig PEC which were composed of 70% of macrophage and lymphocytes and 30% polymorphonuclear cells as indicator cell population. Many centers doing MIF have termed the procedure as macrophage migration-inhibition. Söborg (1967) has used human peripheral leukocytes and Clausen (1972) has also used human polymorphonuclear cells as migratory cell populations.

In the 5 day direct migration systems (Result D, Table V) a fairly pure population of lymphocytes which are also the stimulating and responding cells, has been used as the indicator for migration and its inhibition. There are obvious advantages of the direct migration technique over the indirect migration technique but this will be discussed later in this chapter. Using the direct migration technique, 5 day UMLC cells were mixed in 1 : 1 ratio with fresh leukocytes; this system still demonstrates migration inhibition (Table X). This phenomenon demonstrates that (1) the cultured cells were still viable, (2) they were still able to produce MIF, and (3) the MIF was inhibitory not only to the sensitized cultured cells but also to fresh leukocytes. The last point correlates with early work by David (1964) that a proportion of cells producing MIF can inhibit other cells. The data from the 5 day direct migration technique is consistent with the view that MI obtained from MLC is not significantly different from MI obtained from UMLC (Table V). It is apparent that both the direct and indirect migration techniques are not quantitative enough to distinguish MIF produced by one or two cell populations.

In MLC between unrelated individuals MIF is present in the supernates by the 5th day but not by the third day (Figure 1 and 2) (Table VI, Table VII, and Table VIII) as demonstrated by the indirect migration technique. In the case of multiparous women who have cytotoxic antibodies against their husbands' HL-A antigens, MIF is detectable in the supernates of the cultures between husband and wife (HW) by the third day (Table XI). Significant MIF activity is not detectable on the third day in the supernatant of MLC between cells from the wife and the third control (CW) (Table XI) with the exception of Zackowski in which case the wife reacted with the control who had W17. Antibodies against W15 which the husband had could be absorbed by W17 (Appendix D). A vigorous specificity study should use C and H cells with increasing sharing of HL-A antigens. This might lead to a point where CW supernatants would become positive at day 3. This study would also shed some light into the understanding of other histocompatibility loci. To date this study has not been done.

In the presensitized combination (husbands and multiparous women) MIF production has commenced earlier thus explaining the detectability by day 3. Alternatively MIF production might commence simultaneously in the sensitized and control combinations but the production could be much greater in the cultures where the sensitive wife's cells are confronted with the sensitizing histoincompatible antigen of the husband. In either case one might expect that on the 5th day the presensitized culture (HW) should have greater inhibitory effect as compared with the supernatant fluid of the relevant control culture (CW). This expectation is not fulfilled as shown in Table XI.

Both sets of cultures have significant migration inhibition and the difference between the two MI is not significantly different ($p < 0.1$). This may mean that the sensitivity of the indirect migration technique is not sufficient to permit detection after an optimum amount of migration inhibition material has been produced. Alternately in the presensitized culture (HW), production of MIF may commence, peak and end earlier so that by the 5th day very little fresh MIF is produced. In the control culture (CW) on the other hand, MIF production could commence later and may have barely peaked around the 5th day, so that the total amount of MIF in the supernatants of these cultures (CW) at that time may not be significantly lower than in the presensitized culture (HW). One way to study the problem is to remove the supernatants on the 3rd day and add fresh medium into each set of culture. If there is MIF activity in the fresh medium of 5 day MLC then the production of MIF is continuous and the technique is definitely not sensitive enough to distinguish between 3 day production of MIF in HW and 1 day production of MIF in CW culture.

MIF has been shown to be a product of cells entering the mitotic cycles, more specificially that of the 'S' phase (Tubergen *et al*, 1972). It is of interest to determine if a significant correlation could be obtained between the degree of lymphocyte transformation and the MIF activity of the supernatant fluids in these cultures. In 5 day MLC and UMLC there is always significant blastogenesis whether the cultures are in pooled human AB serum or FCS (Table II, Table III, Table V). However, in all these experiments in which MIF activity is also demonstrable, the BI does not closely correlate inversely with MI (Results B and D). A significant increase of blastogenesis is not followed by a lower MI in the same culture. Thus, although there is a failure to demonstrate MIF activity by the indirect migration technique in 3 day MLC and UMLC, there is significant blastogenesis in these cultures (Table VI, Table VII and Table VIII). BI is an earlier indication of MLC sensitization than MI between unrelated individuals.

When the BI of the unrelated set (CW) is compared with that of the presensitized culture (HW) there is no statistically significant difference either on the 3rd or 5th day in either MLC or UMLC (Table XI). Thus under the experimental conditions, lymphocyte transformation as quantitated by tritiated thymidine uptake does not detect presensitization which is demonstrable by the earlier appearance of MIF activity in the supernatant fluid of HW cultures.

The lack of correlation between blastogenesis and MIF production in MLC may be due to different specificity of stimulation or even due to different subpopulations of cells. Rocklin (1973) has demonstrated that in conditions which allow for preferential elimination of a population of proliferating lymphocytes, there is a population of cells that remains capable of producing MIF. The activity of MIF produced by these lymphocytes is comparable to that of the original population. This suggests that nondividing cells contribute significantly to MIF production whereas the dividing cells are responsible for blastogenesis.

Lymphocyte transformation and proliferation in a 5 day MLC were for a while presumed to be due to HL-A disparity. Recent work by Bach and his colleagues (1972) have shown, however, that there is a lymphocyte defined locus which is demonstrable by MLC. The correlation between the average migration inhibition and the number of HL-A discrepancies in skin graft individuals has been reported "striking and statistically significant" (Falk *et al*, 1970). But it is apparent that the MIF specificities are not restricted to the HL-A system since 3 pairs of unrelated HL-A identicals demonstrate significant migration inhibition in 5 day MLC (Table XII).

The properties of MIF have been reviewed in great detail by David (1971) and Remold (1971) and will not be discussed here. To demonstrate that the migration inhibition phenomenon is due to MIF and not MIF-like-activity, leukocytes from three unrelated individuals have been allowed to migrate in the same supernates, and they all show significant inhibition in 5 day MLC supernates of unrelated individuals (Table IV) and no inhibition whatsoever in 3 day UMLC. This confirms that MIF would inhibit leukocyte migration nonspecificially (David, 1964). When the 5 day MLC cells are mixed with fresh leukocytes, again there is significant inhibition (Table X), similar to David's finding that only a proportion of migratory cells need to be the sensitized cells producing MIF (David, 1964).

In the concentrated supernatants, migration inhibition is not due to decreased viability of migrating cells, since there is no significant difference in the viability of cells migrating in the control supernatants from unmixed cultures vs those in the MLC supernatants (Table XIII). The osmolarities of the supernatants in the same experiment were similar so that there was no possibility that inhibition was due to osmotic differences.

The MIF activity of the supernatant fluids is unaffected at 56°C for 30 minutes. Unheated the mean migration index of five 5 day MLC supernatant was 0.35 ± 0.08 s.e. after heat treatment the mean activity was 0.43 ± 0.13 s.e. MIF has been demonstrated to be heat stable but cryolabile (Bloom & Bennett, 1971). The MI of HW supernates prior to freezing at -70°C for 3 day was 0.44 ± 0.09 s.e. and after freezing the MI was 1.14 ± 0.12 s.e. (Table XIV).

The disadvantage of the indirect migration technique is the lengthy period of incubation and another 2 days are required to concentrate and dialyse the cell free supernates before peripheral leukocytes from another person are allowed to migrate. Besides the procedure requires more than 50 × 10^6 lymphoid cells to accumulate sufficient supernatants. The direct migration technique (Table V) eliminates these difficulties.

Instead of a 3 day MLC incubation period, inhibition by the direct migration technique has further been shown after only 6 hours of incubation of the supposedly presensitized wife's cells and her husband's cells. In this 6 hour direct migration technique 20 out of 21 HW combinations had a MI of less than 0.80 (Table XIII). In all these 21 cases the multiparous women had had cytotoxic antibodies 2 days after delivery against a panel of cells as well as having them against their husbands' antigens at the time when MIF activity was determined (6 weeks postpartum). Two out of 21 CW combinations also had inhibited migration. In both of these two cases the control (C) shares HL-A antigen with the husband (H). Over all there were 7 occasions in which the C shared

HL-A antigens with H.

In other instances when the wives had had cytotoxic antibodies detectable after delivery but none measurable when the experiments were done, 3 out of 5 cases demonstrated migration inhibition in HW combinations (Table XIV). With multiparous women who never had had any detectable antibodies against their husbands or the 5 cell panel 4 out of 8 mixed HW cell populations were inhibited from migration (Table XV). When the wife has cytotoxic antibodies against her husband's cells, direct capillary migration of HW cells nearly always detected MIF activity. The MIF activity in HW cell mixtures is only sometimes observed in those women who have lost cytotoxic antibodies or when the antibodies were not detectable. Thus CMI as demonstrated by the direct migration technique can be induced by gravidity in multiparous women, and can also persist after the disappearance or in the absence of cytotoxic antibodies.

The same phenomenon can also be demonstrated by allowing the cells to migrate in agarose instead of coming out from the capillaries as in the direct migration technique. This agarose migration technique which requires fewer cells than the direct migration technique has been reported to be more sensitive (Clausen, 1972). A comparison study has shown that the 6 hour direct agarose technique is as sensitive as, if not better, than the 6 hour capillary migration technique (Figure 9, Table XVIII).

WH cells that show inhibition of migration in both the direct capillary migration test and the direct agarose migration technique do not demonstrate MIF activity in the presence of the autologous serum of the multiparous women (Figure 9, Table XVI). Thus, blocking phenomenon in the MIF test is specific because autologous serum from one multiparous woman does not affect migration inhibition of another HW cell combination (Table XX). Nonspecific factors in the sera adhered to the placenta may interfere with CMI (Currie *et al*, 1968, Riggs *et al*, 1971) but they cannot take part in this *in vitro* assay. Other nonspecific factors that can interfere with CMI reaction have been demonstrated in maternal sera (Kasakura, 1971) but these factors usually disappear 2 days postpartum and most of the MIF blocking was demonstrable 6 weeks after delivery.

Using these sera in HW UMLC, there is significant inhibition of response as compared to HW cultures with AB serum alone (Table XIX). Although it has not been possible to demonstrate presensitization by changes in blastogenesis it would be interesting to compare sera that cause MIF blocking and UMLC inhibition with a view to antigen specificity and site of inhibition.

The results (Table XIX) show that there is no MIF blocking and UMLC inhibition in two of the thirteen HW combinations and in none of the thirteen control experiments. There are both blocking and inhibition in eight of the other eleven couples. Thus there is discrepancy between the two tests in three out of twenty-six (11.5%). It is possible that excess antibodies are acting on the husband's cells preventing both the release of MIF and the transformation of the wife's cells.

But in the case of central enhancement where antibody-antigen complexes are presumably in smaller quantity than free antibodies it is possible that these complexes are (1) acting on the same recognition site, responsible for the production of MIF and blastogenesis, (2) very close recognition sites that the blockade of one would by steric effect prevent the other recognition site to be in contact with the sensitizing cells, or (3) two far apart recognition sites covered by two different antibody-antigen complexes which happen to be in the serum. The postulate of close recognition sites seems most likely because of the correlation of the two tests and the discrepancy (11.5%) between the tests does not point to the first possibility.

A further analysis of MIF specificity is to compare MIF and cytotoxic antibodies as well as other *in vitro* tests for CMI, such as CML and CI (Figure 10, Figure 11). The percentage (30.5%) of multiparous women (3 or more pregnancies) having cytotoxic antibodies 2 day postpartum compares well with the finding of 33% by Payne and Rolfs (1958). The specificities of the cytotoxic antibodies as determined by a panel of 5 cells cannot be accepted as in any way adequate. However, the reactivity of multiparous sera against such a small panel is not inconsistent with the HL-A profile of husband and panel cells. Moreover when 6 multiparous sera were further typed against a panel of 100 cells or more the cytotoxic antibodies of these sera did not lie outside the HL-A specificities determined by the panel of 5 cells (Table XXIII).

MIF correlates with the cytotoxic antibodies by the Chi square test ($p < 0.01$) in the study of dialysis patients (Table XXVIa). There is also significant correlation between MIF and CML in the dialysis patients as well as multiparous women (Table XXVa and Table XXIVa). In these 2 studies there are 26 positive reactions in which one of the three *in vitro* tests of CMI is positive. MIF is the single positive test in only 4 instances (6.8%). On the other hand, there is reason to believe that single positive reaction by CML or CI alone (22 of 58 positive test or 48%) may not represent CMI.

These studies show that migration inhibition as demonstrated by the agarose technique correlates to some degree with cytotoxic antibodies and CML. Further MIF blocking by immune serum correlates with UMLC inhibition. The MLC response was initially presumed to measure the disparity between the HL-A system (Bach, 1966) by the finding in man that cells of siblings failed to stimulate each other approximately 25% of the time whereas cells of virtually all unrelated individuals tested stimulated. Later studies (Bach *et al*, 1972) indicate that histocompatibility differences which lead to MLC activation are at least in some cases separable from the serologically defined HL-A loci. Eijsvoogel had demonstrated that MLC activation determined by a locus (loci) distinct from the presently known HL-A loci was prerequisite for *in vitro* induction of CML (1972). Recently the same author has shown a CML negative reaction in a MLC positive situation (Eijsvoogel *et al*, 1972). If the present findings on MIF activity do have an *in vivo* significance it could be postulated that MIF activation may be determined by an independent locus which is very close to the HL-A, CML, and MLC loci.

Like the direct capillary migration assay, the direct agarose migration technique has only demonstrated that the migration inhibition is due to the presence of tissue specific antigens which presumably stimulate the presensitized cells to produce MIF activity. In the indirect migration technique the properties of MIF in the supernates can be demonstrated by heating, freezing, chromatography , CsCl gradient and gel electrophoresis separation. Because the supernates are not used in the direct capillary migration assay and direct agarose migration technique, some authors have suggested that inhibition in this system may be due to cytophilic antibodies.

To demonstrate that cytophilic antibodies are not causing MIF-like activity in the direct and agarose migration inhibition assays rabbit antihuman sera have been added in three concentrations in other identical sets of cultures. When the HW cells are inhibited from migration the same HW cells which have been incubated together for 6 hours with rabbit antihuman sera also show MIF activity. Rabbit antihuman serum under these experimental conditions do not prevent inhibition of cell migration, and this makes it unlikely that antibody is the cause of migration inhibition (Figure 12).

In this study MIF activity is demonstrable from the supernates of cells which are used for direct and agarose migration inhibition assay. MIF has been described to have a molecular weight between 67,000-23,000 depending on the species from which it is produced (David, 1972, Valentine & Lawrence, 1973). MIF activity, as demonstrated by guinea pig PEC is shown in G-4 and G-5 fractions of HW cultures supernates (Figure 13, Table XXVII). The G-4 fraction contains molecules of 70,000-25,000 daltons, and the G-5 fraction contains molecules, like the chymotrypsinogen A marker of 23,000 daltons. Human MIF has been reported to have a molecular weight between 40,000-23,000 (Bloom & Bennett, 1972). It is quite probable that the G-4 and G-5 fractions from HW supernates contain the molecule MIF.

WH cultures that show inhibition of migration in both the direct capillary migration and agarose assays do not demonstrate MIF activity in the presence of autologous serum of the multiparous woman. The G-4 and G-5 fractions from the HW culture and autologous serum (HW + WS) do not have any MIF activity (Table XXVII). This could be due to blocking factor(s) in the autologous serum preventing the effector cells to release MIF. Presumably the blocking factor acts either to cover up all the histocompatibility antigen on every sensitizing cell or acts on the much smaller specifically sensitized responder cell population. Unfortunately there is no evidence on either postulate.

The less satisfactory results of serological typing in the selection of unrelated donors (in organ transplantation) may well be explained by the high chance of incompatibility for the lymphocyte defined alleles, which may trigger lymphocyte activation. The MIF technique has been developed from indirect migration to direct migration and then to the agarose technique. All these techniques have shown that inhibition of migration is due to MIF activity. CMI as demonstrated by the MIF techniques can be induced by pregnancy in multiparous women and by transfusion in haemodialysis patients. The MIF test can detect CMI after the disappearance of cytotoxic antibodies; unlike MLC, it can detect presensitization; unlike CML it can detect blocking factors; unlike CI, it takes less than 36 hours to obtain the results. It can be used as a monitoring system for organ transplantation, tumor immunity and autoimmune diseases, and it may be the only CMI test that can detect sensitivity to a certain histocompatibility locus. The MIF phenomenon may be caused by the interaction of an extremely small number of sensitive T cells in contact with specific antigen leading to a series of events that produce a large mononuclear reaction *in vivo* with a heightened activity to destroy "foreign" tissues. The capacity to produce a large cellular reaction is the most economical method by which the organism might maintain effective cellular immunity to a wide variety of antigens. Therefore there is urgent need for further studies of the MIF phenomenon to answer some of the problems existing in immunology today.

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APPENDIX A

The method for the detection of cytotoxic antibodies and determination of HL-A profile is as follows:

Lymphocytes were prepared by obtaining concentrated buffy coat, after the red cells were removed by agglutination and the polymorphonuclear cells by passing through glass bead columns. All antisera (0.001 ml per well) were dispensed into microdroplet testing trays. To prevent evaporation, 0.005 ml mineral oil was added to each well with a multiple needle dispenser. After 0.001 ml of lymphocyte suspension was added into each well, they were mixed and incubated for 30 minutes at room temperature. Then 0.005 ml rabbit complement was added into the mixture. After a 60 minutes of incubation at room temperature, 0.003 ml of 5% aqueous eosin was added to each well and after 2 minutes 0.008 ml of formaldehyde was added.

The results were read by a microscope and the reading of

8 = strong positive 9 - 100% of cell killed

6 = positive-definite drop in viability in comparison to controls

4 = doubtful decrease in viability over control but on positive side

2 = doubtful decrease in viability over control but on negative side

1 = negative-same viability as control

0 = invalid

APPENDIX B*

The method for the detection of "cell mediated lympholysis" (CML) is as follows:

Lymphocyte suspension was prepared from heparinised blood by Ficoll-isopaque separation, washed once in Hank's solution. Contaminating erythrocytes were lysed with sterile distilled water for 5-10 seconds, then the preparation was rapidly brought back to near isotonicity with the addition of more Hank's. This treatment did not interfere with the viability nor the function of the lymphocytes as effector cells whereas treatment with NH_4Cl-Tris for the same purpose markedly crippled lymphocytes to act as effector cells. After one more wash the effector lymphocytes were resuspended in TC 199 with 10% fetal calf serum (FCS) and adjusted to a final concentration of 10^6 cells/ml. The target lymphocytes were labelled with ^{51}Cr: 5-10 × 10^6 cells in 0.1 ml with TC 199 with 10% FCS were incubated with 50-100 µCi of ^{51}Cr as Sodium Chromate (Amersham, Toronto) at 37°C with shaking for 60 minutes, then washed 4 times with TC 199 with 10% FCS at 4°C and adjusted to a final concentration of 2 × 10^6 cells/ml in TC 199 with 10% FCS.

All tests were done in duplicate in 12 × 75 mm. plastic tubes in which one ml. of effector cell suspension and 0.025 ml of target cell suspension were mixed. After mixing the cells, the tubes were centrifuged at 200 G for 5 minutes to bring the cells in close contact before incubation at 37°C in 5% CO_2 atmosphere for 4 hours. The reaction was then stopped by adding one ml. of cold Hank's to each tube. After centrifugation at 400 G for 7 minutes, the supernatant was decanted into another tube and supernatants and cell pellets counted in a gamma spectrometer. ^{51}Cr release was calculated by the formula:

$$\% \, ^{51}\text{Cr release} = \frac{\text{cpm in supernatant}}{\text{cpm in supernatant + cell pellet}} \times 100$$

APPENDIX C

The method for the detection of "colony inhibition" is as follows:

Fibroblast lines were obtained from skin, and tested in the microcytotoxicity assay (Hellstrom 1966). All cultures were grown and subcultured in Eagle's minimum essential medium (MEM) containing 10% heat inactivated fetal calf serum. For testing, fibroblasts used had been subcultured for a maximum of 3 days. One hundred cells, in 10 µl of medium were dispensed into each of 96 wells in a Falcon microtest tissue culture plate and 100 µl of MEM added. After 18 hours incubation the supernatant medium was removed and 100 µl samples of 1:5 dilution of the serum to be tested for blocking effects, or control serum, were added. After these additions, the plates were incubated for 30 minutes at 37°C in a moist atmosphere of 4-5% CO_2. Next the medium was decanted, and lymphocyte cytotoxicity was assessed by adding 2×10^5 effector cells from experimental and control samples to each well, giving a ratio of 1:2000 target to effector cells.

Following 48-72 hours of incubation, the plates were stained, washed and counted. Inhibition was expressed as a percentage of the fibroblasts exposed to control lymphocytes so that 100% inhibition is complete disappearance of all fibroblasts. Results were also analysed by Student's "t" test.

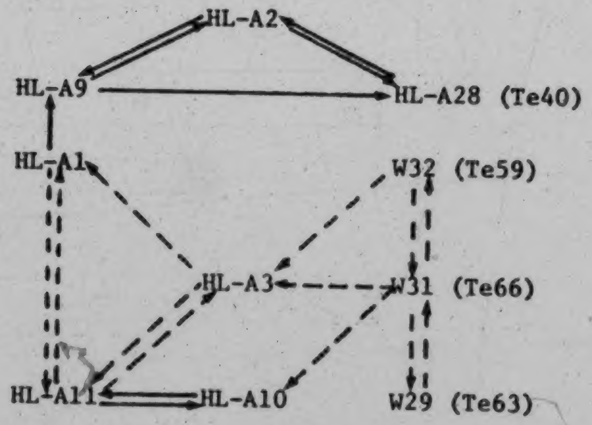
APPENDIX D*

ANTIBODY————→ANTIGEN WHICH ABSORBS IT

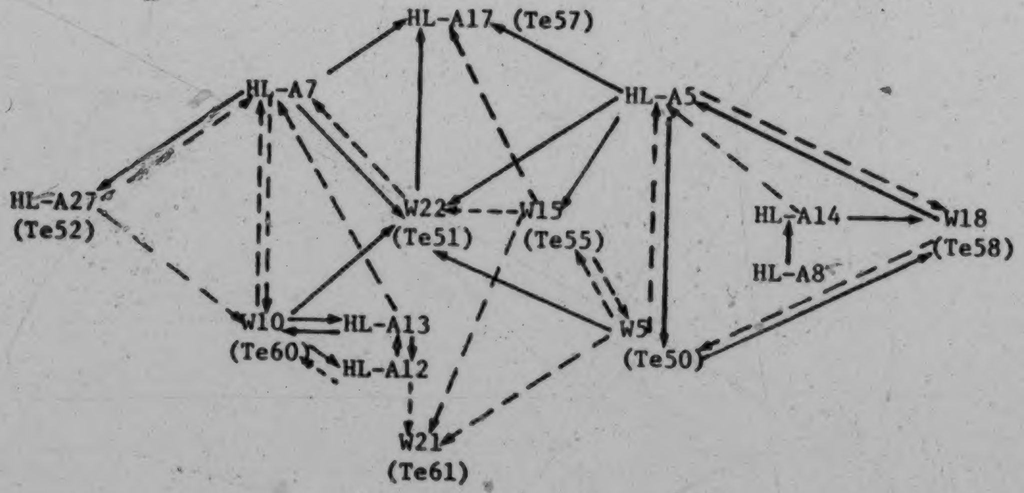
————→COMPLETE ABSORPTION

– – – – – →PARTIAL ABSORPTION

HL-A SEGREGANT SERIES 1


HL-A2
HL-A9
HL-A28 (Te40)
HL-A1
W32 (Te59)
HL-A3
W31 (Te66)
HL-A11
HL-A10
W29 (Te63)

HL-A SEGREGANT SERIES 2


HL-A17 (Te57)
HL-A7
HL-A5
HL-A27
(Te52)
W22
(Te51)
W15
(Te55)
HL-A14
W18
(Te58)
HL-A8
W10
(Te60)
HL-A13
HL-A12
W5
(Te50)
W21
(Te61)

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*Mittal, K.K. and Terasaki, P.I. (1972) Cross reactivity in the HL-A system. *Tissue Antigen 2:* 94, and personal communication.

APPENDIX E*

HL-A - Based on Histocompatibility 1972
and PIT's letter of July 1, 1972
1st Segregant Allelic Series; or LA

	HL-A or Workshop (W) numbers	1	2	HLA28	3	11	9		10		W19	W29		W30	W31	W32
							W23	W24	W25	W26		(19.1)	(19.2)	(19.3)	(W19.4)	(19.5)
EQUIVALENT SIMILAR	Terasaki	(Te1)	(Te2)	Te40	(Te8) (Te3)	(Te13)	(Te4)		(Te12) W10.1	W10.2	Te19	Te63		Te66		Te39
	Kissmeyer-Nielsen Thorsby	LA1	LA2	Ba*	ILN LA3	ILN*	LA4		KH							
	Dausset	DA11	DA1 "XAC" DA2	DA15	DA12	DA21	DA27	DA32 (DA16) (DA3)	DA29	DA28 (DA17)		DA22	DA25	DA25' (DA26)	DA25" (DA33)	
OR	Others	LA-1 Te-8 AO-19 Lc-1	Bt-5 Lc-2	Ao5 Lc17	To10		Lc12	BIM Ao35 Bt-9 To12	To31	Bt-8 To40		Ao77 Bt15		Lc21	LA-W Lc26.1	Ao28 To30

2nd Segregant Series; or '4' Series

	HL-A or Workshop (W) numbers	5	W5	W18		W15	HLA 17	7	W22 [22.1 22.2]	HLA 27	8	HLA 14	12	13	W10	W16	W21	W4 4a	W6 4b
EQUIVALENT SIMILAR	Terasaki	(Te11) (Te6)	Te50 (Te5)	Te58 (Te18)		Te55 (Te15)	W 17 Te57 (Te17)		Te51 (Te22)	W 27 Te52 (Te14)		W14 Te34 (Te16)	(Te9)	(Te26)	Te60	Te64	Te61		
	Kissmeyer-Nielsen Thorsby	4C KI AS-AJ AS*	R 4C	R* 4C*	SL	LND-AJ LND (6 antigens) LND*	SL	7 AA*	AA AA-AJ	FJH-AJ FJH FJH*	8	Mak1	KN12 T12	HM	BB	U18	ET ET* SL-ET		
	Dausset	DA5	Da20		DA19	DA23		DA10			DA8	DA18	DA4			DA31	DA26	DA3	DA7
OR	Others	Ao12 4C Bt25 Te5	4C AO13	4C* Bt20		AJ	Ao70 MApl Bt12	Ao2 4d	To28 W22-AJ W22*	To29 W27AJ W27*	Bt-2 Te-7	Mak1 To27	Ao15 To11 4a11	HK Bt23 To21	To23	Ao81		4a Ao27	4b Ao72

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*Dossetor, J.B. (1973) Personal communication.